UNIVERSITY OF NORDLAND

MASTER THESIS

Mitogenomic analyses of the two cold-water octocorals *Alcyonium digitatum* and *Primnoa resedaeformis*

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Alisa Kravtcova, May 2015.

Abstract

Mitochondrial genome sequencing is an active and productive field in current research of animal species. The application of Next-Generation Sequencing techniques has significantly improved the process retrieving valuable molecular data.

In this study, the mitogenomes from two cold-water octocorals from the north Atlantic region (Norwegian waters) were completely sequenced, using the IonTorrent PGM technology. While one species (*Alcyonium digitatum,*L. 1758) represents the shallow water soft corals, the other species (*Primnoa resedaeformis,* L. 1812) is a deep-sea gorgonian species. Thus, according to classic taxonomy these two octocoral species are expected to be distantly related.

The resulting mitogenomes are in the line with previous research of related species and are similar in gene content and order to the inferred ancestral type of mitogenome organization in octocorals. At the same time, several interesting sequence features were explored. Deviations from the common pattern among octocorals are expressed in nucleotide sequence heterogeneity and intergenic space structure.

Phylogeny analyses highlighted the relationships of these species based on whole mitogenome sequences of all available octocorals. Here, the two studied species group together, and forming a separate branch among octocorals. This observation is very surprising since *Alcyonium digitatum* and *Primnoa resedaeformis* represent soft corals and gorgonians, respectively. These interesting results provide a basis for further studies of mitochondrial genomes of cold-water octocoral variation in both species.

List of abbreviations

As	Antisense
atp6	F0 ATP synthetase complex, subunit 6 gene
atp8	F0 ATP synthetase complex, subunit 8 gene
Bp	Base pair(s)
cox1	Cytochrome oxidase, subunit 1 gene
cox2	Cytochrome oxidase, subunit 2 gene
cox3	Cytochrome oxidase, subunit 3 gene
cob	Cytochrome b gene
DNA	Deoxyribonucleic acid
etc	et cetera
GTR	General Time Reversible
IGR	Intergenic region
IPTG	Isoprpyil β-D-1-thiogalactopyranoside
Kb	Kilobase(s)
ML	Maximum Likelihood
Met	Methionine
Msh-1 (mtMutS; mutS)	Mutation Supressor Homolog 1; mitochondrial mutation supressor gene
mtDNA	Mitochondrial DNA
nd1	NADH dehydrogenase, subunit 1gene
nd2	NADH dehydrogenase, subunit 2 gene
nd3	NADH dehydrogenase, subunit 3 gene
nd4	NADH dehydrogenase, subunit 4 gene
nd4L	NADH dehydrogenase, subunit 4L gene
nd5	NADH dehydrogenase, subunit 5 gene
nd6	NADH dehydrogenase, subunit 6 gene
NGS	Next-generation sequencing
Nt	Nucleotide(s)
ORF	Open reading frame
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
RPM	Rounds per minute
rRNA	Ribosomal RNA
rnl	Ribosomal large subunit gene
rns	Ribosomal small subunit gene
Sec	Second(s)
tRNA	Transfer RNA
trnM	Transfer RNA (f-Met) gene
Xgal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

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Introduction

Cnidaria

Cnidaria is a taxon of special interest to biologist. The phylum Cnidaria is a basal group of animals that originated early in the metazoan evolution *Proceedings of the National Academy of Sciences*. Phylum consists of approximately 9000 species with main groups represented by Anthozoans, Cubozoans, Staurozoans, Hydrozoans and Scyphozoans. Despite of their ancestry, cnidarians exhibit high morphological plasticity and variability in the reproductive traits and life cycles (McFadden et al., 2001). They are simply organized animals with little bilateral symmetry. Some animals possess sack-like body and are sessile while others have an actively swimming medusa lifeform. While diverse life forms can be observed in this taxon, all animals inhabit marine environments with a few exceptions of freshwater-dwelling organisms (for instance, freshwater hydra *H. oligactis*).

In contempt of their morphological simplicity, cnidarians are the key species in some types of ecosystems. Particularly, corals shelter up to one-third of marine fauna species (Plaisance et al., 2011), provide unique conditions to microorganisms, and are hot points of biodiversity in marine and oceanic environments (Sunagawa et al., 2010).

Corals are among the most prominent organisms within this taxon. They form a class Anthozoa with two main assemblages – Hexacorallia and Octocorallia, respectively. Both groups consist mostly of colonial forms that participate in reef-building by creating dense beds on the ocean's floor. Therefore, both groups contribute to creation of important benthic ecosystems.

Mentioned subclasses are different in their morphology (Daly et al., 2007). Octocorals have eight-fold symmetry and simple tissue organization. They are composed of mesoglea, forming a dense matrix, and continuous epidermis, connecting the whole colony. An outer tissue, called coenenchyme, is often solidified. Body plan also includes eight mesenteries and eight tentacles. Colonies are polymorphic in their color. Animals prefer different habitats, but usually it is depths in the range of 3 to 50 m (Moen, 2004).

Hexacorallia is a well-studied group (63 complete mitogenome sequences available in GenBank) while the importance of Octocorallia was reflected in studies more recently (23 complete mitogenome sequences available in GenBank). Moreover, tropical species are best studied and described (Iguchi et al., 2012; Shinzato et al., 2011; Stanley Jr, 2003; Weis et al., 2008), thus species from another locations are interesting objects for further investigations.

Genomic studies of coral genomes are very promising in many respects: molecular techniques applied to species from unusual life conditions can result in obtaining new valuable information, molecular tools (such as primers, markers, etc.) and products (drugs, GFP-like proteins, toxins and venoms) as well as valuable information about diversity of basic animal groups.

Figure 1. A) An example of *Alcyonium digitatum* presented in yellow and white morphs

B) An example of a part of *Primnoa resedaeformis*

Photo: A) JC Schou B) Leo Shapiro   Both images are taken from eol.org

Mitogenome research

The complete sequences of mitochondrial genomes have become a useful tool in current research on cnidarians (Chapman et al., 2010; Kayal and Lavrov, 2008). Compared to variably organized and complex nuclear genomes, mitogenome sequences are relatively easy to assemble and annotate. Moreover, the mitochondrial genome contains a stable set of genes and is readily amenable to comparative analyses. After first mitogenomes have been sequenced and assembled, a cascade of molecular data, describing mitochondrial DNA (mtDNA) organization and functioning, appeared. A variety of feature characteristics of the mitogenome has recently been uncovered, and these studies improved our basic knowledge of organelle genome (Emblem et al., 2014; Shao et al., 2012). The broad use of mtDNA makes it a suitable marker in population studies and phylogeny inferences in detecting SNPs, variability, and selective sweeps in coding sequences. Research on mitochondrial genome structure also contributes in exploring nuclear genomes since it represents a first step in the understanding of organism function at a basic level.

Application of Next Generation Sequencing (NGS) has revolutionized the current field of research (Miller et al., 2011). However, a combination of NGS and different techniques in mitogenome research is probably the most powerful approach. This because it, will ensure both efficient processing and quality of the sequences. Thus, NGS reads can be successfully combined and verified with techniques such as PCR, molecular cloning and Sanger sequencing (Johansen et al., 2010).

Such comprehensive data can be used to characterize marine populations, species and communities, as a basis of nature conservation strategies, and establishment of protected marine areas (Shinzato et al., 2011). This becomes more important as the anthropogenic impact on the environment is increasing. Marine habitats contain prominent ecosystems threatened by human induced exploitation (underwater mining, fishery, etc.), pollution, ocean acidification processes, and climate change (Hofmann et al., 2008). New molecular data will also facilitate blue biotechnology, bioprospecting and its vast propagations in pharmaceutics and therapy (Bruckner, 2002; Cho et al., 2009; Otero-González et al., 2010).

Therefore, the application of genomics supported by NGS in marine species research is of high priority.

Previous knowledge about octocoral mitogenomes

There are no complete nuclear genome sequences of octocorals up to date, but previous studies in other cnidarians revealed unexpected complexity of cnidarian nuclear and mitochondrial genomes (Beagley et al., 1995; Chapman et al., 2010). Indeed, specific features are abundant within this phylum in either genomes. Here, focus will be mainly on the mitogenome since it is an object of interest.

Anthozoan mitogenomes are usually organized in a circular DNA molecule with the size range from 16 to 25 Kb in hexacorals and 18-19 Kb in octocorals. Those species which have linear DNA can also have several mitochromosomes of different size. However, hexacoral and octocoral genomes have noticeable differences in both gene content and genome organization. Both genomes contain typical 13 essential protein-coding genes, coding for proteins involved in oxydative phosphorylation processes, 2 ribosomal RNA subunits and a transfer RNA (f-Met), which is common for both subclasses. In addition, hexacoral genomes have homing endonuclease gene, another transfer RNA gene (Tryptophane) and group I introns.

Octocoral mitochondrial genome composition is highly conserved, but gene order is often rearranged. It consists of typical 14 protein-coding genes and specific gene *msh-1* (Mutation Supressor Homolog 1; *mtMutS*), found only in octocorals. Genes are usually separated by intergenic regions (IGR) – short non-coding sequences of up to 100 nucleotides. Some more details are presented in the Table 1.

Table 1. Mitochondrion genome content in two Anthozoan subclasses

Mitogenome feature	Octocorallia	Hexacorallia
Size (Kb)	18-19	16-25
Topology	circular	circular
Protein coding genes	14	14
rRNA genes	2	2
tRNA genes	1	2
HEG	No	Yes
Introns	No	Yes
msh-1	Yes	No

Idea and realization of the project

The chosen species are very promising candidates for mitogenome sequencing study. Both species are common cold-water soft corals from the North Atlantic region, that belong to the Alcyonacea – the order in the Octocorallia subclass. While *Alcyonium digitatum* ("Dead men's fingers") is a soft coral within the family Alcyoniidae found in shallow waters, *Primnoa resedaeformis* belongs to the deep sea gorgonian family Primnoidae. Taxonomically these species are expected to be distantly related octocorals, but little molecular data about are available in databases to challenge the relationship analysis.

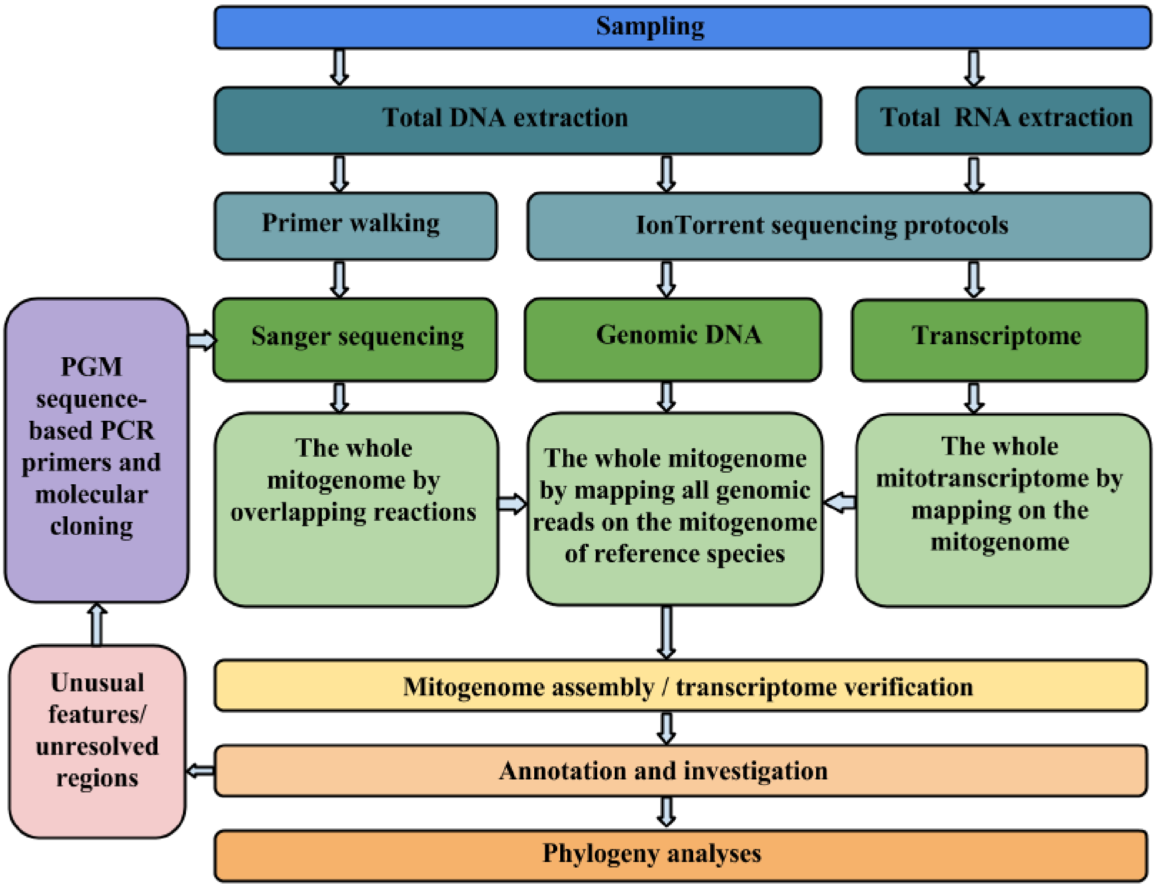
The present study applied Ion semiconductor sequencing technology to mitogenome and mitotranscriptome sequencing of two octocoral species (Fig.2). For the purpose of verifying the results obtained by IonTorrent sequencing, we used Sanger sequencing amplified mtDNA regions.

Molecular cloning procedures were chosen as an additional approach that would also improve the resolution of the sequence. This technique is effective for verification of poorly resolved parts of the mitogenome since it is able to produce clean sequencing data and improved coverage of problematic DNA regions. Transcriptome sequencing output is used for verification of gene sequences as well as identifying abundant transcripts. Thus, we were able to obtain a high quality mitogenome sequence from both study species.

The acquired mitogenomes were used together with the set of available published octocoral mitogenomes species for the reconstruction of phylogenetic relations.

The use of Ion semiconductor sequencing has also a key advantage because a pool of whole genomic DNA reads that is created during preparation procedures. These reads can be used for the further studies.

Figure 2. Workflow scheme of experiments in the present study.



Materials and methods

Sample collections

Live *P. resedaeformis* samples were collected at a *Lophelia pertusa* reef at Nord-Leksa, Norway (63º36'N;9º24'E) at 150-200 m depth using the ROV Minerva, RV Gunnerus (NTNU, Trondheim). Samples were stored in absolute ethanol at -20ºC for DNA extraction. Samples for RNA extraction were homogenized in TRIzol and frozen at -80ºC, or stored in RNA*later*® RNA Stabilization solution (Life Technologies™) at -20 ºC in order to prevent RNA degradation.

Live *A. digitatum* samples were collected by scuba-divers at Mørkvedbukta Research station (67°16'N; 14°33'E), Bodø, Norway at 3-5 m depth. Samples were preserved in absolute ethanol at -20ºC for DNA extraction. Samples for RNA extraction were frozen at -80ºC, or stored in RNA*later*® solution and frozen at -20 ºC in order to prevent RNA degradation.

Nucleic acid isolation

Coral tissue samples (2-5 mg) were mechanically homogenized in 2 ml MagNa Lyser Green Beads Tube (Roche) with Precellys 24 homogenizer (Bertin Technologies™) at 10000 rpm until complete homogenization. Total genomic DNA was extracted with Epicentre MasterPure™ Complete DNA and RNA Kit (Illumina™) and Urea protocol (see Appendix). Both protocols exploit broad range specificity Proteinase K. The Urea protocol also includes phenol/chloroform and chloroform/isoamyl extractions together with ethanol precipitation. The Epicentre kit contains manufactured protein precipitation agents.

Standard TRIzol protocol (Chomczynski, 1987), modified for cod (MG group) was used to extract total RNA from both fresh tissue and frozen samples. Standard requirements for work with RNA were considered as previously described (Nielsen, 2011). RNAseZap®Solution (Life Technologies™) was used to clean working surfaces from RNAses.

For the purpose of measuring the amount of nucleic acid in the probe and the purity of sample, several basic methods were used. Qubit™ dsDNA BR Assay kit (Invitrogen™) and High sensitivity RNA Assay kit (Invitrogen™) protocols were used directly after extraction procedures to assess approximate amount of material to work with.

Qubit ® 2.0 Fluorometer (Invitrogen TM) measurements are based on the fluorescence of the probe after binding with fluorescent agents. Nanodrop® ND-1000 (Thermo Fisher Scientific™) device was used to analyze concentration and purity of the sample. This device exploits a ratio of different wavelengths as a standard value of a pure sample. Agilent 2200 Tape Station System (Agilent Technologies) device was used to assess length of molecules and sample molarity after shearing and amplifying as well as other procedures, when proceeding to library and template preparations. Genomic DNA Screen Tape and High Sensitivity RNA Screen Tapes were used.

All measurements were done according to manufacturer's instructions and with negative control sample.

PCR

Only parts of protein coding genes of the studied species mitogenomes are available in online databases. Therefore, PCR primers were constructed using these sequences and also using multiple alignments of different octocoral species sequences. These octocoral-specific primers were combined randomly (except using forward and reverse primer for one gene in the same reaction) in PCR reactions. The strategy was to obtain amplification of different regions and find an overlapping reactions whose products would cover the whole mitogenome.

Primers used for PCR and Sanger sequencing reactions are listed in the Appendix B. PCR kit from TaKaRa (TaKaRa Bio Inc.) was used when preparing master mix. TaKaRa *LaTaq* Polymerase was chosen because of abilities to amplify long amplicons, as well as its proofreading properties (TaKaRa Bio Inc., 2004). Reaction mixture is presented in the Table 2. All preparation steps were performed on ice.

Table 2. Reaction mixture for PCR

Component	Amount, µl
DNA sample	1
Primer, F	1
Primer, R	1
LA Taq Polymerase	0,2
dNTP mix	4
Mg2+ Buffer	2,5
Millipore water	15,3

Thermocycling conditions were as following: initial denaturation at 94ºC for 5 minutes, 25 cycles of 94ºC for 30 seconds, 55ºC for 30 seconds, 72ºC for 4 minutes and final elongation at 72ºC 4 minutes. Annealing temperatures were set according to the melting temperatures (Tm) of the primers. Reactions were run with a negative control in order to check for contamination.

Visualization by agarose gel

DNA fragments were separated on 1% agarose gel (1 g of Ultra-pure Agarose (Invitrogen™) per 100 ml of 0,5 TBE buffer) with SYBR® Safe (Invitrogen™) (4 µl per 100 ml). 1 Kb+ DNA Ladder (Invitrogen™) was used to determine size of products. PCR products were mixed with Blue Bromophenol 6x loading dye (1 µl of dye per 5 µl of product) before loading into wells. Products were visualized using Gel Logic 200 Imaging system (Kodak™) and Safe Imager™. Qiagex II Gel Extraction Kit (150) (Agarose Gel Extraction protocol) (Qiagen) kit was applied for products purification from gel.

Sanger Sequencing

Successfully amplified products were Sanger sequenced (BigDye v.3.1) to verify sequences difficult to determine by IonTorrent. PCR primers were diluted 10-fold for the use in sequencing reactions. The reaction mixture is presented in the Table 3. Thermocycling conditions were as follows: initial denaturation at 96ºC for 5 minutes, 25 cycles of 96ºC for 10 seconds, 50ºC for 5 seconds, and 60ºC for 4 minutes. Reaction was performed at the Molecular Biology Lab, UiN, Bodø and samples were shipped to UiT/UNN afterwards where were processed on a 3130 xl GeneticAnalyzer® (Applied Biosystems™).

Table 3. Reaction mixture for Sanger sequencing

Component	Amount,µl
PCR product (gel extracted)	2
Big dye enzyme	1
Big dye Buffer 5x	1
Forward/Reverse primer	3
Nuclease-free water	3

Ion Torrent sequencing protocols

E-Gel® electrophoresis system was applied for size selection when preparing a template to further steps. E-Gel is a compact device that uses ready-made and stained E-Gel® SizeSelect™ Agarose Gels (2%), and works as a usual electrophoresis chamber. This procedure helps to select a part of library with desirable size.

Real-time quantitative PCR (qPCR) procedure was done in order to calculate the exact amount of molecules ligated with adapters from both ends. This procedure was done using StepOnePlus Real-Time PCR system (Life Technologies™) and Ion Library Taqman Quantitation kit.

Emulsion PCR was performed with Ion One Touch™ 2 System with Ion PGM™ Template OT2 200- and 400 kits (Life Technologies™) and checked with Qubit Quantitation Assay (Life Technologies™) kit.

Whole genome sequencing was done by using Ion Torrent™ PGM and 316 v.2 sequencing chips. Ion Torrent Low Input Protocols were used because of low (50-100 ng) extraction output from nucleic acid isolation procedures. Sequence quality was assessed based on the sequencing run report and manual inspection of tracer.

Low Input RiboMinus™ Eukaryote System v2 kit (Ambion™) protocol and MicroPoly(A)Purist Kit protocol were used to purify samples from rRNA and enrich PolyA RNA, respectively. Ion Total RNA Seq kit v2 was used to convert RNA into cDNA by reverse transcription reaction and to prepare a template for further work. Ion One Touch™ kits for 200- and 400 bp and 316 v.2 sequencing chips were used. Quality control was performed by analyzing the sequencing summary. All procedures were performed according to user manuals from manufacturers.

Bioinformatics

FinchTV (Geospiza Inc.) was used for the quality score inspection of Sanger sequenced DNA fragments as well as the length.

CLC Genomics Workbench (Qiagen™) software was used as a basic bioinformatic tool for further analyses. Mapping of all reads on mitogenome of the reference species sequences was done in order to sort out all nuclear reads. *C. rubrum* mitogenome was used as a reference for

P. resedaeformis and *P. resedaeformis* subsequently as a reference for *A. digitatum*. Both length and similarity fractions were set to be equal 0.8 in the purpose of increasing robustness. The resulted mitochondrial reads were used for further mitogenome *de novo* assembly based on overlapping parts of these reads. MITOS webpage assembly tool (http://mitos.bioinf.uni-leipzig.de/) and MITObim script (MITOchondrial Baiting and Iterative Mapping) (Hahn et al., 2013) were used for further verifications of genome assembly and annotation. The latter is a MIRA assembler based Perl-script that requires no mapping on a reference mitogenome. The *cox1* gene was used as a setout for assembly with "--quick" option. Mapping of all protein coding genes from complete octocoral mitogenomes (see Appendix D) available in GenBank was done for detection of protein coding sequences. Multiple alignments with the same set of species and genes were built then to evaluate reading frames, assess quality of assembly and annotate a mitogenome. Reading frames were also verified with EMBOSS Transeq web page and CLC Workbench with all 6 reading frames and Mold Mitochondrial genetic code settings. European Bioinformatic Institute resources (ebi.ac.uk) and NCBI (ncbi.nlm.nih.gov) resources were also used to detect similarity between sequences (BLAST algorithms), and to translate sequences into proteins, obtain sequences from databases.

Molecular cloning

Cloning was performed for the purpose of amplification of irresolute regions detected after the assembly. In this study *mutS* gene sequence was cloned since this gene is very variable and needs auxiliary sequencing techniques. Primers were made based on PGM sequencing results and listed in the Appendix. These 10-fold diluted PCR primers were used to amplify DNA fragments coding for *mutS* gene. PCR reaction conditions were the same as those for ordinary reaction (see PCR). Gel electrophoresis on 1% agarose gel was done then for excising products. Gel purification step was performed with Qiagen kit (see PCR). Samples were frozen in Eppendorf LoBind® Tubes (Eppendorf™) at -20 ºC then until further procedures.

Topo® TA Cloning® kit for sequencing and One Shot® Top 10 Competent Cells (Life Technologies) were used to perform transformation reaction – an insertion reaction where amplified PCR product is introduced in bacterial genome by a vector. The amounts of PCR product in transformation reactions were 2 and 4 µl in order to get the most suitable amount of colonies. All growth media were prepared according to user guides and manuals.

Kanamycin (50 mg/ml) was used as an antibiotic agent for selection of insert-containing vectors. Either reaction was made in a 50 ml tube filled with 45 ml of LB medium with 45 µl of kanamycin added. Each reaction was triplicated on separate plates with amount of S.O.C. medium-combined cells of 50, 100, and 150 µl, respectively. 40 µl of Xgal and 40 µl of IPTG (100 mM) were added directly on agar plates. Ampicillin was used as antibiotic agent for negative control. To confirm insertion of gene into bacteria PCR was performed directly on bacteria clones using M13 primers. Standard 1% agarose gel electrophoresis was performed to visualize the result.

White colonies were collected using a pipet tip and transferred into LB-medium and incubated in a Multitron Standard Incubation Shaker (Infors HT™) at 150 rpm in 37 ºC overnight. Cultures were transferred in Eppendorf LoBind® Tubes (Eppendorf™) and plasmids were purified with PureLink® Quick Plasmid Miniprep Kit (Invitrogen™) according to manufacturer's protocol.

Purified products were prepared to Sanger sequencing. Reaction mixture for Sanger sequencing reaction: 5µl of purified plasmid DNA, 3 µl of M-13 sequencing primer, 1 µl of BigDye 5x sequencing buffer (Applied Biosystems), 1 µl of BigDye 3.1 enzyme and nucleotide mix (Applied Biosystems). Reaction was performed in GeneAmp 9700 thermocycler with following settings and then shipped to UiT/ UNN, Tromsø (see Sanger sequencing).

Phylogenenetic analysis

The present study focused on the relations of the species under investigation within the octocoral class. Two different datasets were used for estimating distance between the studied species and all available octocoral mitogenomes.

First dataset includes a concatenated alignment of all *mutS* genes+corresponding genes from the studied species. This approach was used for estimating distance between sequences of *mutS* gene since it is a specific sequence presented only in mitogenomes of octocorals.

Second dataset is a re-annotated mitogenome where the protein coding genes were sequentially concatenated in a following order: *atp6* and *atp8* genes, *cox1-3* genes, *nd 1-4*, *4L*, *5-6* genes, *mutS* and *cob*. These reconstructed full-length mitogenomes were used in multiple alignments and building phylogenetic trees. Intergenic regions and ribosomal genes were omitted in analyses. *C. granulosa* was used as outgroup for both datasets since taxonomy of this species is somewhat contradictory and places this species outside Octocorallia. Alignments, distance and model tests and ML phylogenetic trees were done in the CLC Genomics Workbench (Qiagen™) software package as well as distance and model tests. Common model was GTR+G+T. Bootstrapping value in ML trees was 1000.

Results

Coral samples, preservations and extractions

Tentacles, epidermal tissue, and coenenchyme were used for nucleic acid extraction procedures. Both extraction methods were suitable for DNA isolation from the coral tissues but notable difference in the output was observed. The Epicentre kit was used for DNA extraction and further library preparation of *P. resedaeformis* samples. The Urea protocol was used for DNA extraction from *A. digitatum* samples because the Epicentre kit gave overshearing (Fig.3) of DNA and insufficiently short fragments (based on size-selection results).

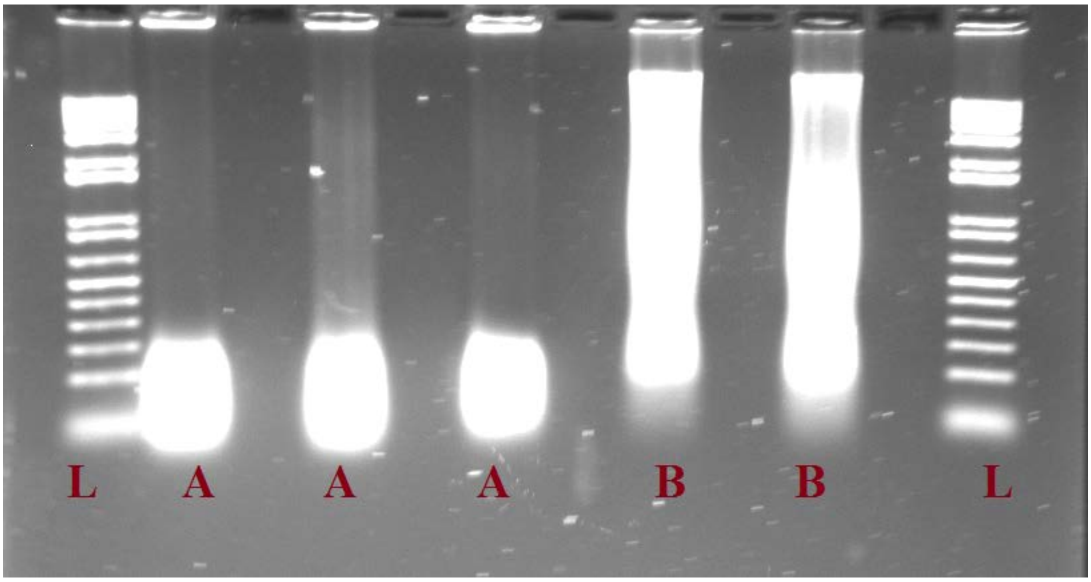


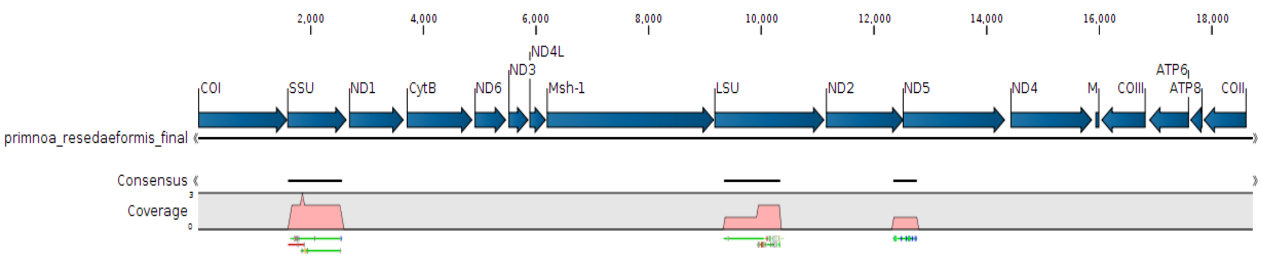
Figure 3. Agarose gel analysis of total DNA isolated from *A. digitatum* with two different methods - the Epicentre kit (lanes A) and the Urea protocol (lanes B). DNA size ladder (L).

PCR and Sanger sequencing

Fragments of different sizes were amplified from DNA of both species, successful primer combinations are listed in the Appendix B. Most amplified products were obtained from *P. resedaeformis* DNA, and few were gained from *A. digitatum* DNA. The largest product sizes were fragments of 1.5 to 2 kb in length which were subjected to Sanger sequencing. A total of 41 DNA fragments were Sanger sequenced. Obtained sequences were in the range of 200 bp to 1200 bp in sizes, and cover different parts of protein coding regions and RNA genes. However, length and quality scores of reads were appropriate and sufficient only in 12 fragments amplified from *P. resedaeformis* DNA and 2 from *A. digitatum*. Sanger sequebces with low quality scores were removed. The most amplified regions are sequences between forward and reverse primers for *nd4L*, *rns* and *rnl* genes.

We initially intended to cover the whole mitogenome with overlapping reactions, but this approach resulted only in amplification of several fragments of the mitogenome. However, sequenced fragments are identical to corresponding regions in the whole mitogenome sequences and give strong support to Ion Torrent sequencing results (Fig.4.).

Figure 4. Mapping of all Sanger sequenced fragments on linearized view of the mitogenome of *P.resedaeformis*



Ion Torrent PGM sequencing

DNA sequencing

Two new mitochondrial genomes were sequenced in this study. The complete mitogenome sequences are presented in Appendix D. Fragments of approximately 400 bp (Appendix C) were obtained during the size-selection procedure when preparing the *P. resedaeformis* libraries. The total number of reads obtained for *P. resedaeformis* was 2.8 million (2.765.810) (Appendix C), which corresponds to a coverage of 146 times. Furthermore, the mitogenome was found to have a GC content of 36.6 %. Fragments of approximately 400 bp were obtained during the size-selection procedure when preparing the *A. digitatum* libraries. The complete mitogenome was sequenced using 316 v 2 chip. Similar to that of *P. resedaeformis,* the *A. digitatum* sequencing generated 3.0 million reads (3.018.931) with a mitogenome coverage of 159 and GC content of 37%.

Mitotranscriptome sequencing

Sequencing of the *P. resedaeformis* transcriptome resulted in 25.947 reads with a mean read length of 67 bp. Furthermore, the chip 25 %, the poly - clonality was 36 % and usable reads only 18%. In comparison, the *A. digitatum* transcriptome sequencing was much more efficient. Here, 1.2 million (1.200.348) reads were obtained, with mean read length of 155 bp. Chip loading and usable reads were twice as much (54% and 35%, respectively), and the poly - clonality significantly lower (28%).

Assembly and annotation of mitogenomes

Contigs of 18.726 bp and 18.790 bp were obtained from *P. resedaeformis* and *A. digitatum*, respectively, and represent the complete mitogenomes. Assembly was also performed using the MITOBIM script, with identical results. However, assembly with MITOS gave somewhat different results that include duplicated genes and additional transfer RNA genes not found by the other approaches.

Assembly and annotation revealed surprising similarity between the two mitogenomes. The *A. digitatum* and *P. resedaeformis* mitogenomes are less than 19 Kb, which is a consistent among octocorals. Both mitogenomes contain 14 protein-coding genes (Fig. 5) - 7 Complex I genes (*nad* 1, 2, 3, 4, 4L, 5, and 6), one Complex III gene (*cob*), 3 Complex IV genes (*cox* 1, 2 and 3), 2 Complex V genes (*at*p6 and *atp8*) and a specific octocoral *msh-1* (Mutation Supressor Homolog; mtMutS) gene. Protein-coding sequences are located on both strands and have a typical ancestral (Table 9) octocoral organization: *cox1 - rns – nd1 - cob – nd6 – nd3 – nd4L - mutS - rnl – nd2 – nd5 – nd4 - trnM* (as) – *cox3* (as) – *atp6* (as) – *atp8* (as) – *cox2* (as). Length of intergenic regions is 760 nucleotides in the mitogenome of *A. digitatum* and 648 nucleotides in those of *P. resedaeformis* and fall within the common range for octocorals (414 to 957 bp). The content percentage of the whole mitogenomes is presented in Table 4.

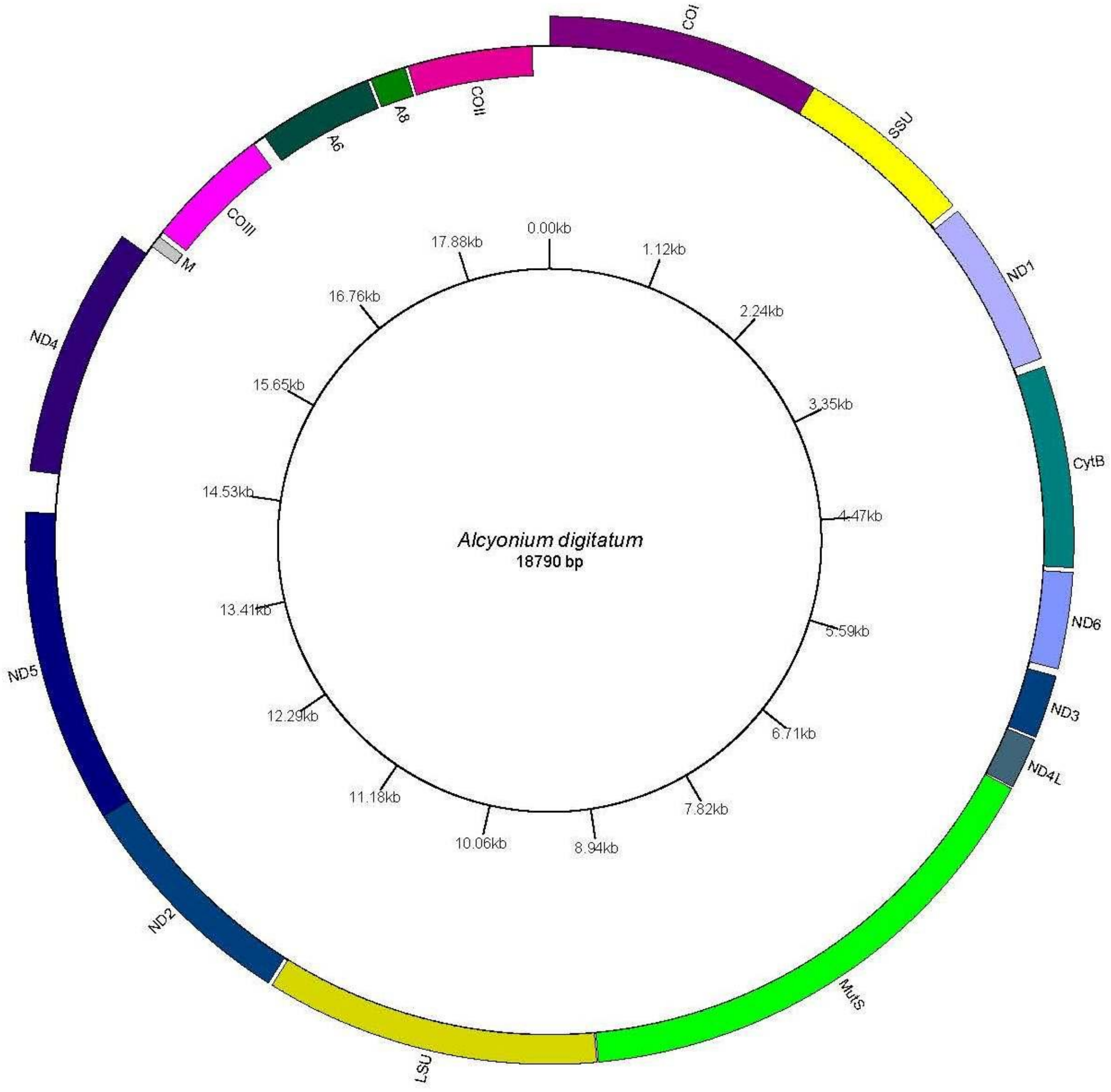
Table 4. Mitogenome composition in studied species

	A. digitatum,%	*P. resedaeformis*,%
Functional genes	74.8	75.2
RNA genes	15.85	16
IGRs	9.35	8.8

Table 5. Annotation of mtDNA genes and intergenic regions (IGRs) in *A. digitatum* and *P. resedaeformis*

Genes and IGRs	*Alcyonium digitatum*, position	Size, nt (aa)	*Sinularia peculiaris NC_018379 Size, nt (aa)*	*Narella hawaiinensis NC_026192 Size, nt (aa)*	Size,nt (aa)	*Primnoa resedaeformis, position*	Genes and IGRs
cox1	1-1582	1582 (527) ATG/T	1582 (527) ATG/T	1597 (532) AGT/T	1582 (527)	1-1582	*cox1*
igr-1	1582	0	0	-6	0	1582	*igr-1*
rns	1583-2624	1042	1051	1126	1052	1583-2634	*rns*
igr-2	2625-2666	42	47	-1	44	2635-2678	*igr-2*
nd1	2667-3635	969 (322) ATG/TAG	969 (322) ATG/TAG	981(326) ATG/TAG	969 (322) ATG/TAG	2679-3647	*nd1*
igr-3	3636-3686	51	27	144	54	3648-3701	*igr-3*
cob	3687-4853	1167 (388) ATG/TAA	1167 (388) ATG/TAG	1161(386) ATG/TAG	1167 (388) ATG/TAG	3702-4868	*cob*
igr-4	4854-4880	27	40	22	40	4869-4908	*igr-4*
nd6	4881-5438	558(185) ATG/TAG	558(185) ATG/TAG	564 (187) ATG/TAG	558(185) ATG/TAG	4909-5466	*nd6*
igr-5	5439-5481	43	55	43	43	5467-5509	*igr-5*
nd3	5482-5847	366 (121) ATG/TAG	354 (117) ATG/TAG	354 (117) ATG/TAG	354 (117) ATG/TAG	5510-5863	*nd3*
igr-6	5848-5865	18	19	28	19	5864-5882	*igr-6*
nd4L	5866-6159	294 (94) ATG/TAA	294 (97) ATG/TAA	294 (97) ATG/TAA	294 (97) ATG/TAA	5883-6176	*nd4L*
igr-7	6160-6172	13	13	28	13	6177-6191	*igr-7*
mutS	6173-9115	2943 (980) ATG/TAA	2973 (990) ATG/TAA	2937 (978) ATG/TAG	2970 (989) ATG/TAA	6190-9159	*mutS*
igr-8	9116-9124	9	9	17	9	9160-9168	*igr-8*
rnl	9125-11061	1937	1968	2211	1948	9169-11116	*rnl*
igr-9	11062-11087	26	31	0	28	11117-11144	*igr-9*
nd2	11088-12449	1362 (453) ATG/TAG	1374 (457) ATG/TAG	1149 (379) ATG/TAG	1374 (457) ATG/TAG	11145-12518	*nd2*
igr-10	12437-12449	-13	-13	-13	-13	12506-12518	*igr-10*
nd5	12437-14254	1818 (605) ATG/TAG	1818 (605) ATG/TAA	1872 (623) ATG/TAG	1818 (605) ATG/TAG	12506-14323	*nd5*
igr-11	14255-14493	239	96	43	97	14324-14420	*igr-11*
nd4	14494-15941	1449 (482) ATG/TAA	1449 (482) ATG/TAA	1449 (482) ATG/TAA	1449 (482) ATG/TAA	14421-15869	*nd4*
igr-12	15942-15998	56	57	62	59	15870-15928	*igr-12*
trnM(as)	15999-16069	71	71	71	71	15929-15999	*trnM(as)*
igr-13	16070-16106	37	39	44	36	16000-16035	*igr-13*
cox3(as)	16107-16872	786 (261) ATG/TAG	786 (261) ATG/TAA	786 (261) ATG/TAG	786 (261) ATG/TAG	16036-16821	*cox3(as)*
igr-14	16873-16954	62	64	47	64	16822-16885	*igr-14*
atp6	16955-17662	708 (235) ATG/TAA	708 (235) ATG/TAA	708 (235) ATG/TAA	708 (235) ATG/TAA	16886-17593	*atp6*
igr-15	17663-17685	23	24	20	23	17594-17616	*igr-15*
atp8	17686-17901	216 (71) ATG/TAG	216 (71) ATG/TAA	216 (71) ATG/TAG	216 (71) ATG/TAG	17617-17832	*atp8*
igr-16	17902-17922	21	22	22	21	17833-17853	*igr-16*
cox2	17923-18684	762 (251) ATG/TAG	762 (251) ATG/TAG	762 (251) ATG/TAG	762 (251) ATG/TAG	17854-18615	*cox2*
igr-17	18685-18790	106	112	112	111	18616-18726	*igr-17*
mtDNA		18790	18742	18838	18726		*mtDNA*

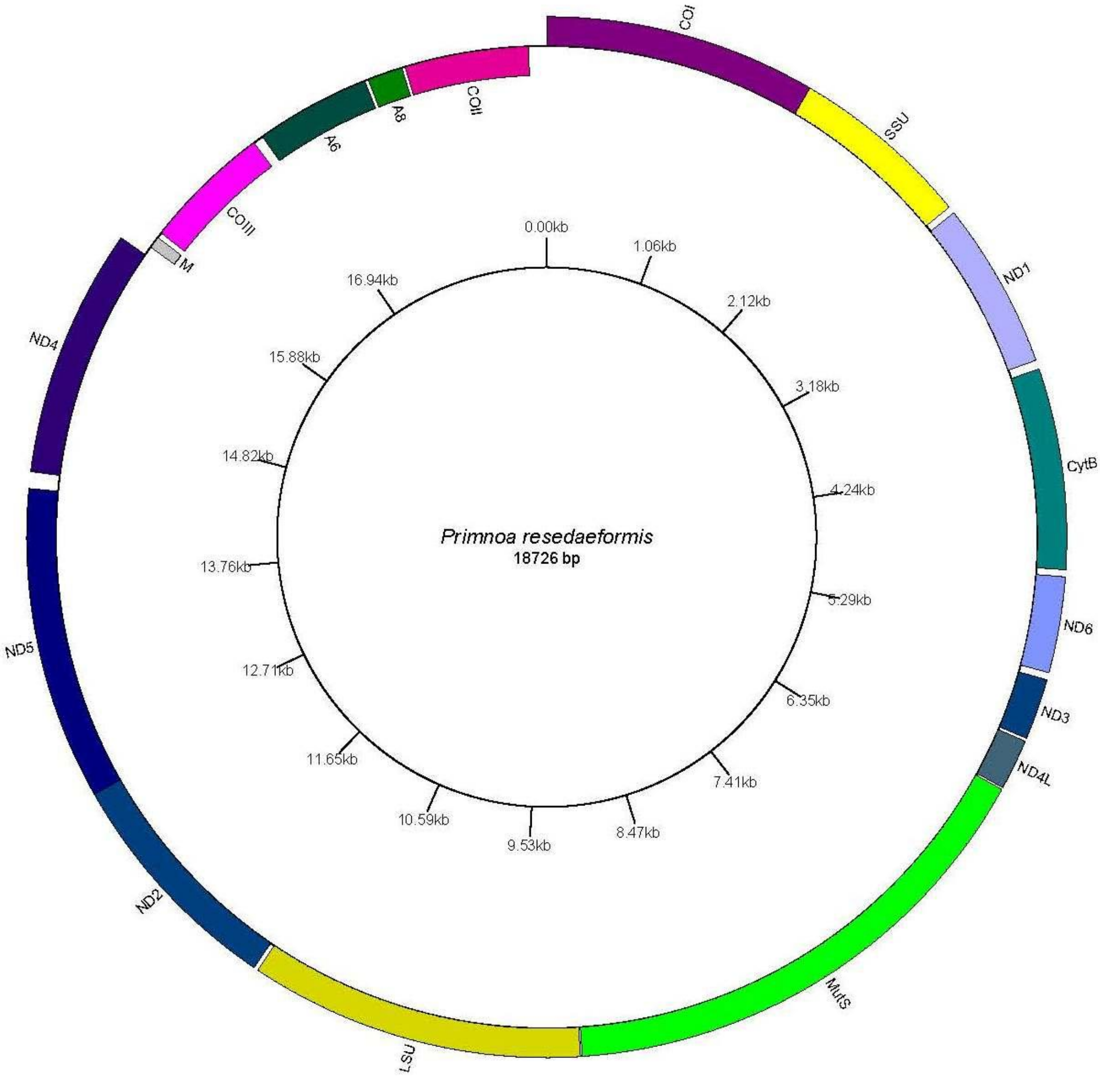
Figure 5 A. Mitogenome organization in newly sequenced mitogenome of *A. digitatum*



A circular view of the *A. digitatum* mitochondrial genome

Blue, Complex I genes, Pink, Complex IV genes, Green, Complex V genes, Emerald, Complex III genes, Bright green, MutS gene, Yellow, rRNA genes. Genes on heavy and light strands are annotated on outer and inner circles, respectively.

Figure 5 B. Mitogenome organization in newly sequenced mitogenome of *P. resedaeformis*



A circular view of the *P. resedaeformis* mitochondrial genome

Blue, Complex I genes, Pink, Complex IV genes, Green, Complex V genes, Emerald , Complex III genes, Bright green, MutS gene, Yellow, rRNA genes. Genes on heavy and light strands are annotated on outer and inner circles, respectively.

Sequence feature analysis

Mitogenome

The major part of the mitogenome comprises protein-coding sequences. Since the function of these proteins is crucially important, there is a high degree of conservation in the nucleotide sequences. A comparison of available octocoral mitogenomes was done in order to detect variability in length nucleotide sequences of protein coding genes. Visual inspection of alignments revealed low variability in the nucleotide and protein sequences as well as in the length of genes.

Complex I genes (*nad1*, *2*, *3*, *4*, *4L*, *5*, and *6*)

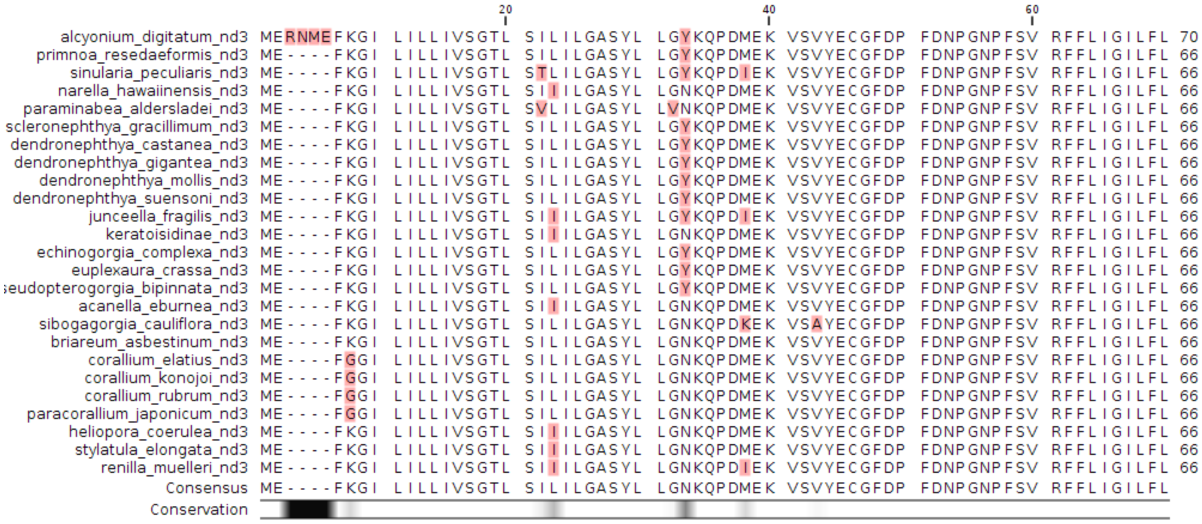
The *nd1* gene is conserved in both nucleotide and protein sequences. Only several nucleotides are found to be different from other octocorals, which give no difference in amino acid sequence since these are synonymous substitutions. The length of the gene is the same in *A. digitatum* and *P. resedaeformis*, and 3 nt (corresponding to one amino acid) shorter than in most of other species (Table 6).

Table 6. Length variability in Complex I genes

Species	*nd1, nt*	*nd2, nt*	*nd3, nt*	*nd4, nt*	*nd4L, nt*	*nd5, nt*	*nd6, nt*
Alcyonium digitatum	969	1362	366	1449	294	1818	558
Primnoa resedaeformis	969	1374	354	1449	294	1818	558
Acanella eburnea	981	1322	348	1449	294	1818	552
Antillogorgia bipinnata	972	1093	354	1449	294	1818	558
Briareum asbestinum	972	1164	354	1449	294	1818	558
Corallium elatius	972	1140	354	1449	294	1818	555
Corallium konojoi	972	1140	354	1449	294	1818	555
Corallium rubrum	972	1140	354	1449	294	1818	555
Dendronephthya castanea	972	1158	354	1449	294	1818	558
Dendronephthya gigantea	972	1039	354	1449	294	1818	558
Dendronephthya mollis	972	1158	354	1449	294	1818	558
Dendronephthya suensoni	972	1158	354	1449	294	1818	558
Echinogorgia complexa	972	1152	354	1449	294	1818	558
Euplexaura crassa	972	1158	354	1449	294	1818	558
Junceella fragilis	981	1122	354	1449	294	1818	555
Keratoisidinae sp. BAL 208-1	981	1320	348	1449	294	1812	552
Narella hawaiinensis	981	1140	354	1449	294	1872	564
Paracorallium japonicum	972	1140	354	1449	294	1818	555
Paraminabea aldersladei	972	1140	354	1449	294	1818	549
Scleronephthya gracillimum	972	888	354	1449	294	1818	558
Sibagogorgia cauliflora	996	1140	354	1449	294	1842	555
Sinularia peculiaris	972	1374	354	1449	294	1818	558
Heiopora coerulea	981	1356	354	1461	294	1818	558
Renilla muelleri	981	1356	354	1449	294	1818	555
Stylatula elongata	981	1383	354	1449	294	1818	555

The *nd2* gene is more variable in size and sequences (Fig. 7). Notable differences are found at the 5'-end of nucleotide alignment *A. digitatum* and *P. resedaeformis*. Both genomes contain a large region of 228 nucleotides that exceeds the gene sequences in most other octocorals. Moreover, this region is invariable between the studied species and is similar to those of more distantly related species (e.g. *H. coerulea*, *S. elongata* and *R. muelleri*). Gaps are found across the alignment as a result of a pronounced nucleotide variations in corresponding gene in other octocorals. The 3'-end is also variable and is the only part of the alignment where there is a significant difference between *A. digitatum* and *P. resedaeformis* (409-416 aa positions in *A. digitatum*). The *nd3* gene also reveals heterogeneity in the nucleotide sequence of *A. digitatum* which has 12 inserted nucleotides at the 5'-end. This region is unique in comparison to other congeneric species. The protein alignment also reflects this difference (see Fig.6) while the other parts in both sequences show conservation in nucleotide and amino acid positions.

Figure 6. A variable 5’-end region in the protein sequence of *nd3* gene in *A. digitatum*



The *nd4* gene length and its amino acid sequence is consistent within octocorals, but some nucleotide variations are still observed. 31 nucleotide substitutions differ *A. digitatum* and *P. resedaeformis* from other octocorals though protein structure is very similar within this class.

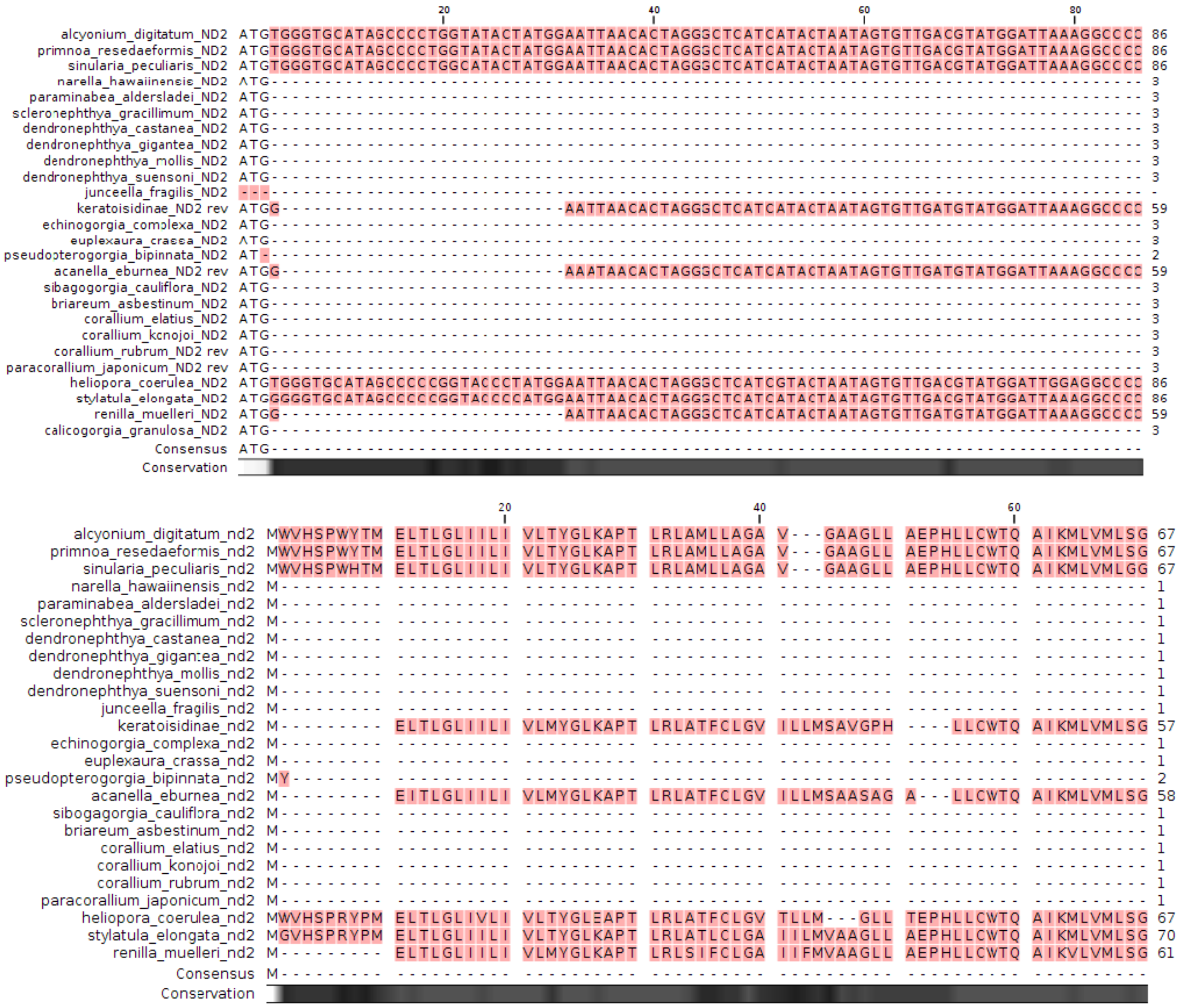
The *nd4L* gene length is the most conserved gene in both length and amino acid sequence among all the octocorals. However, some minor differences on the nucleotide level are still present, resulting on occasional synonymous substitutions.

The *nd5* gene length differs scarcely within octocorals with common size slightly exceeding 1800 nucleotides. Some variable nucleotide positions are met in the gene sequence, but these result in very few amino acids substitutions. Two regions with large gaps are caused the presence of *S. cauliflora* and *N. hawaiinensis*.

Finally, the *nd6* gene shows little and synonymous nucleotide variation resulting in similar amino acid sequences.

Full alignments can be examined in the Appendix E.

Figure 7. An example of the variable 5'-end region in *nd2* gene sequence (nucleotide-upper, protein-lower) in the studied species.



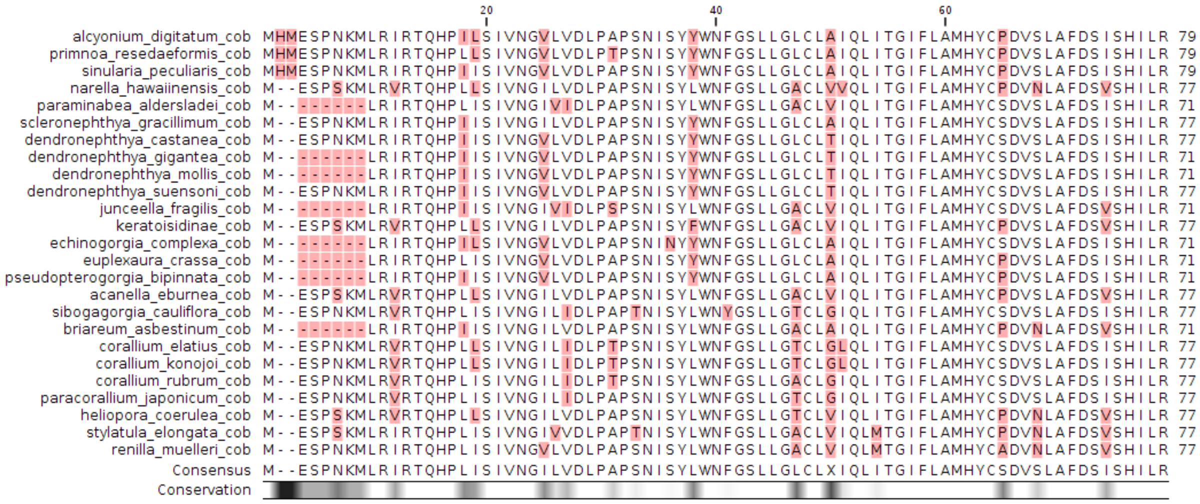
<u>Complex III gene (*cob*)</u>

One of longest genes among octocorals is *cob* which is 1167 in both *Alcyonium digitatum* and *Primnoa resedaeformis* (the longest among octocorals is found in *C. rubrum* and *P. japonicum* in 1194 bp). The nucleotide sequences have similarity between the studied species, but appear to contain more substitutions than other genes. 5'-end heterogeneity is seen in 6 nucleotide positions presented only in *Alcyonium digitatum*, *Primnoa resedaeformis* and *S. peculiaris* and thus making a gap in the nucleotide alignment. 38 nucleotide positions are variable and another

12 are the same in studied species, but different in another octocorals. Aminoacid variation is 16 positions and studied species differ between each other and 8 amino acid positions.

Full alignments can be examined in the Appendix E.

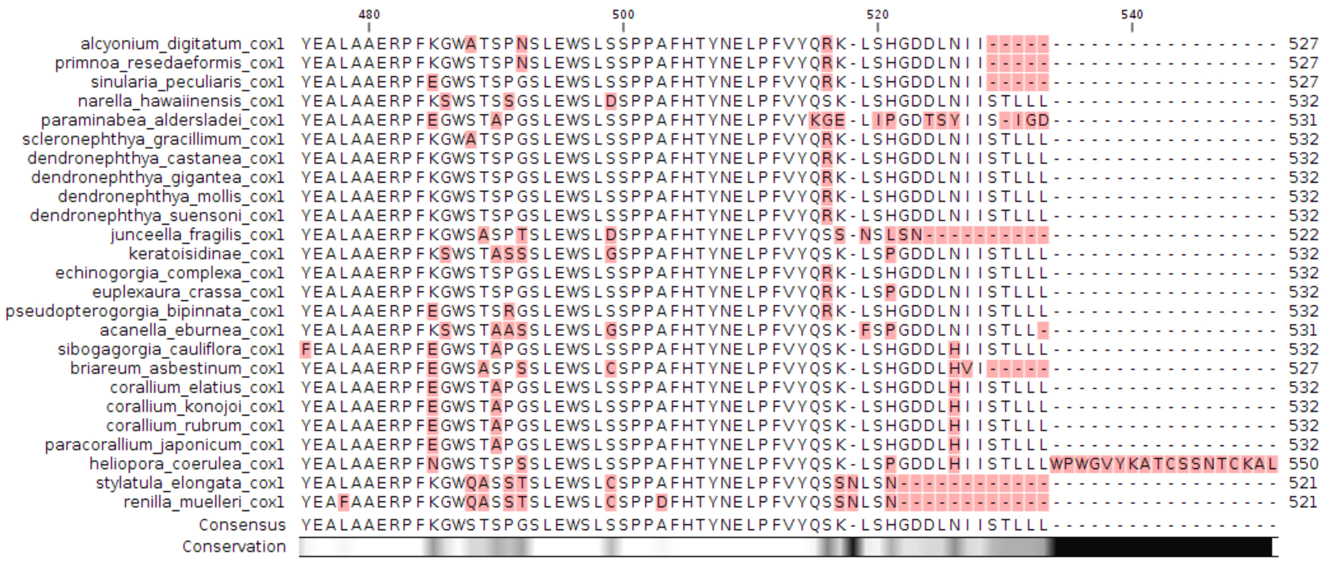
Fig. 8. 5'- heterogeneity in the nucleotide sequences of *cob* in the studied species



<u>Complex IV genes (*cox 1,2 and 3*)</u>

The studied species possess almost the shortest *cox1* genes among octocorals (Table 7). The nucleotide sequence variation is due to 37 transition-transversion events in different positions throughout the gene sequence. Some of these positions contribute to amino acid variations forming a non-synonymous substitutions. As a result, amino acids with uncharged groups are replaced with amino acid with hydrophobic or charged groups. Most of the nucleotide variations fall outside the Folmer region (29-736 bp positions region used for barcoding) leaving it relatively conserved. The amino acid sequence remains conserved, but some heterogeneity is found at the 3'-end (Fig.9). Sequences in both species lack 5 terminal amino acid residues, which is not common for other species.

Figure 9. 3’-heterogeneity in the protein alignment of *cox1* gene.



Incomplete termination codon found in COI gene sequences in both genomes indicating that this region undergoes polyA restoration (RNA editing) in order to be a functional mRNA.

The *cox2* contains 11 variable positions in the nucleotide sequence that reside mainly in the 230-700 bp region of the gene. Several gaps are introduced in the nucleotide alignment of *cox2* gene. They represent differences often found in the sequences of the Isidinae family species and other distantly related octocorals. However, most of the substitutions are synonymous and only two amino acids are different between *A. digitatum* and *P. resedaeformis.* Three other amino acids residues differ in the studied species from the other representatives of octocorals while the overall amino acid sequence is unchanged.

The *cox3* has 24 variable nucleotide positions and each contributes to amino acid sequence change. Eight amino acid residues are different in the studied species and most of variation is represented by changing amino acid with hydrophobic group on those with positive.

Full alignments can be examined in the Appendix E.

Table 7. Length variability in Complex IV genes

Species	*cox1, nt*	*cox2, nt*	*cox3, nt*
Alcyonium digitatum	1582	762	786
Primnoa resedaeformis	1582	762	786
Acanella eburnea	1597	762	786
Antillogorgia bipinnata	1597	762	786
Briareum asbestinum	1582	762	786
Corallium elatius	1597	762	786
Corallium konojoi	1597	762	786
Corallium rubrum	1597	762	786
Dendronephthya castanea	1597	762	786
Dendronephthya gigantea	1597	762	786
Dendronephthya mollis	1597	762	786
Dendronephthya suensoni	1597	762	786
Echinogorgia complexa	1597	762	786
Euplexaura crassa	1597	762	786
Junceella fragilis	1569	762	786
Keratoisidinae sp. BAL 208-1	1597	762	786
Narella hawaiinensis	1597	762	786
Paracorallium japonicum	1597	762	786
Paraminabea aldersladei	1596	762	786
Scleronephthya gracillimum	1597	762	786
Sibagogorgia cauliflora	1597	762	786
Sinularia peculiaris	1582	762	786
Heiopora coerulea	1653	762	819
Renilla muelleri	1566	762	786
Stylatula elongata	1566	762	786

Complex V genes (*atp6* and *atp8*)

The *atp6* nucleotide sequence has a little nucleotide variability of 19 positions and gaps introduced by *C. rubrum* and *P. japonicum.* The amino acid sequence shows more conservation. Only 5 amino acids are variable and are replaced by amino acids with different side chain group only once.

The *atp8* gene sequences are highly similar between *Alcyonium digitatum*, *Primnoa resedaeformis* and other octocoral species and differ with 3 amino acids. Length in both Complex V genes is highly conserved inside octocorals (Table 8).

Full alignments can be examined in the Appendix E.

Table 8. Length variability in Complex V genes

Species	*atp6, nt*	*atp8, nt*
Alcyonium digitatum	708	218
Primnoa resedaeformis	708	218
Acanella eburnea	714	218
Antillogorgia bipinnata	708	219
Briareum asbestinum	708	218
Corallium elatius	708	218
Corallium konojoi	708	218
Corallium rubrum	708	218
Dendronephthya castanea	708	218
Dendronephthya gigantea	708	218
Dendronephthya mollis	708	218
Dendronephthya suensoni	708	218
Echinogorgia complexa	708	218
Euplexaura crassa	708	218
Junceella fragilis	708	218
Keratoisidinae sp. BAL 208-1	717	218
Narella hawaiinensis	708	218
Paracorallium japonicum	708	218
Paraminabea aldersladei	708	218
Scleronephthya gracillimum	708	218
Sibagogorgia cauliflora	708	218
Sinularia peculiaris	708	218
Heiopora coerulea	708	218
Renilla muelleri	708	218
Stylatula elongata	708	218

mutS

This gene has the greatest variability in both nucleotide, protein alignments and size, as seen in the presented alignments. Sequencing revealed that *Alcyonium digitatum* possesses one of the shortest *mutS* genes among octocorals - 2943 bp (the shortest mutS genes are 2937 in Narella and 2940 in) and 2970 bp in *P. resedaeformis.*

Several gaps presented in the nucleotide sequence brought by distantly related species. Number of variable nucleotide positions is 115. The amino acid sequence is also more variable than in other genes. *A. digitatum* amino acid sequence in *mutS* gene is shorter than those of *P. resedaeformis* and 52 amino acid positions are variable (Fig.10) and thus represent interesting features of *mutS* gene in studied species. Protein annotation revealed differences in the length of helices and sheets in the secondary structure (Appendix F) though overall structures remains recognizable in all octocorals.

Function of this gene is still putative and its description is addressed in the Discussion section.

Full alignments can be examined in the Appendix E together with predicted secondary structure scheme.

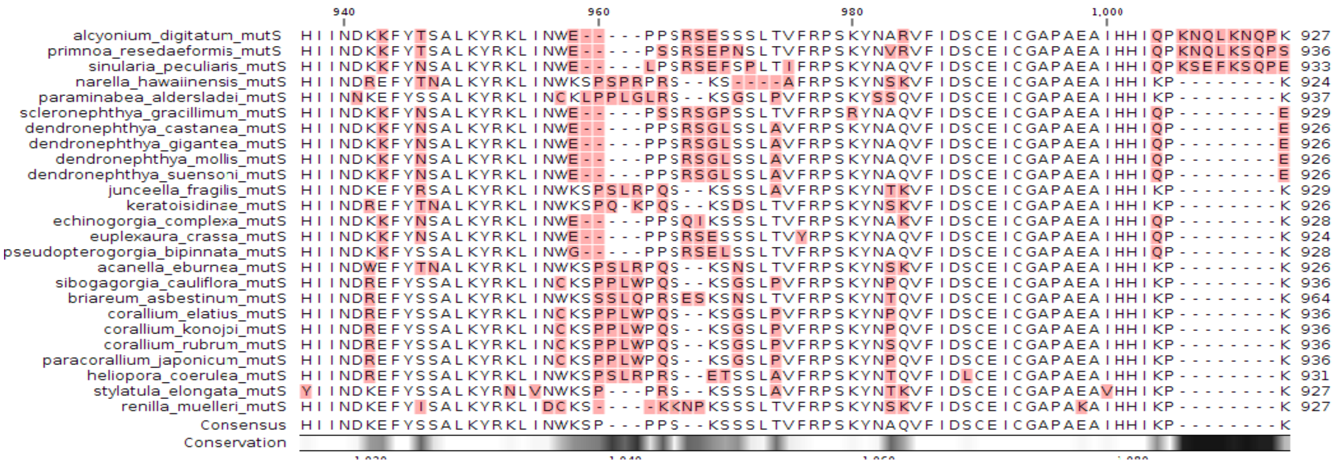


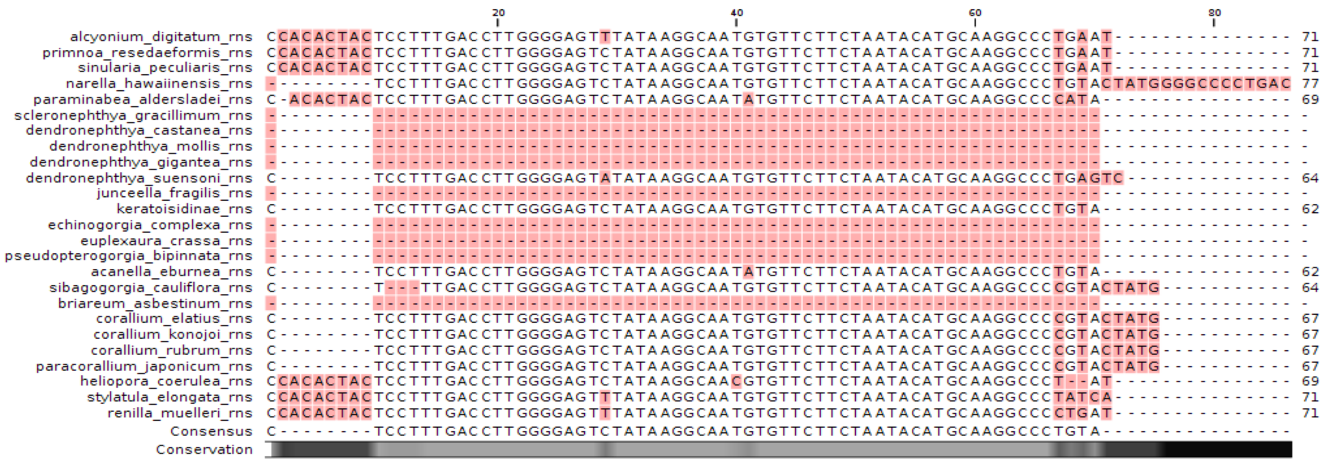
Fig. 10. An example of a variable region in *mutS* protein alignment in octocorals.

rRNA genes

This group of genes is expected to have the most conserved nucleotide sequence throughout the mitogenome since their function is crucial to the organellar translation machinery. Multiple alignments support these expectation in general but several interesting features are discovered at the same time.

Length variation in *rns* is 28 nucleotides between *A. digitatum* and *P. resedaeformis*. Nucleotide sequences are very similar with only one region with pronounced variation (720-740 bp position). Several gaps are introduced into the alignment by *N. hawaiinensis* and *B. asbestinum* highlighting distance between species. Sequence of *A. digitatum* has several deletions in different regions across the sequence.

Fig. 11. A region of the sequence variability in *rns*.



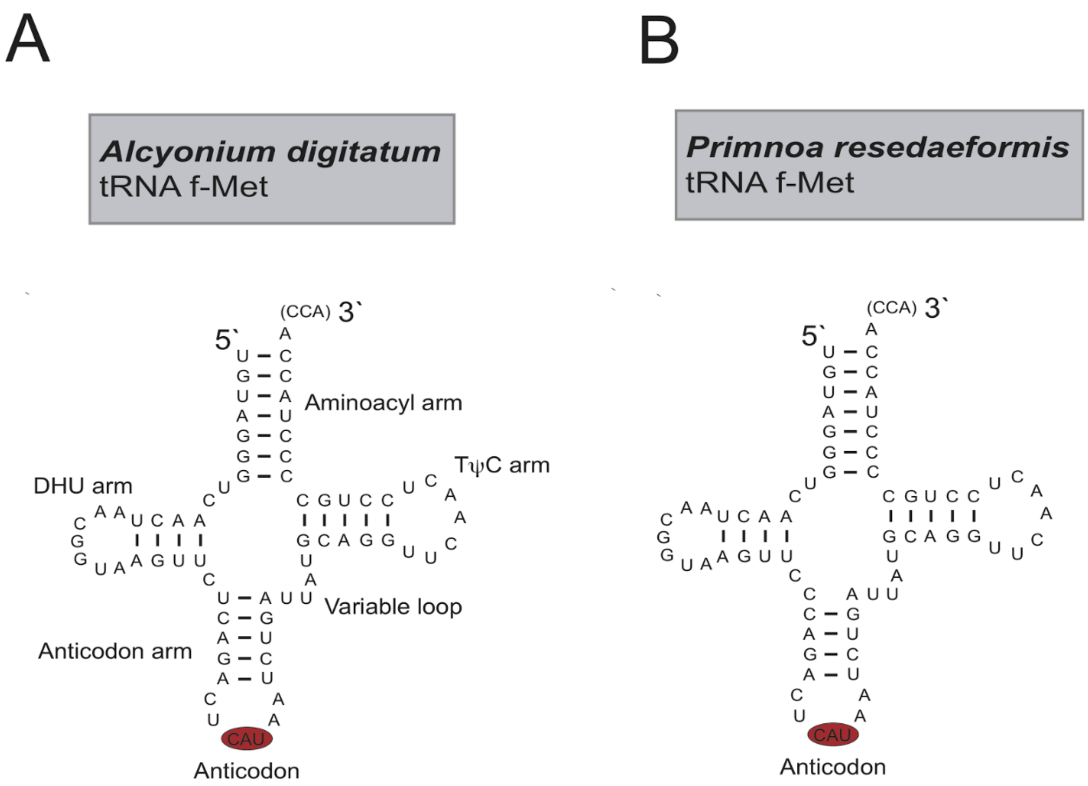
Despite of expected level of similarity, nucleotide sequences of *rnl* possess interesting features. First, there are several large gaps of 25-60 nucleotide positions in different regions of the gene. As it is presented in the alignment, these gaps brought mainly by distantly related species.

There are frequently occurred nucleotide positions that are deleted in the *A. digitatum* sequence.

A. digitatum has the shortest *rnl* sequence among the octocorals (1937 bp), and those corresponding gene in *P. resedaeformis* is 1948 bp.

tRNA f-Met sequence is highly conserved among octocorals. Only one nucleotide difference is found between (in the anticodon arm) in *P. resedaeformis* and *A. digitatum*, which is also reflected in a highly secondary structures (Fig.12).

Figure 12. Predicted secondary structure of tRNA f-Met in the studied species



In general, variabilities in the gene sequences are mainly located in the 5'-end or 3'-end heterogeneities. These differences are by transitions - transversions events, as well as insertions and deletions. Most variations in nucleotide sequences include *mutS*, *cob* and *nd2* genes. The longest gene is *mutS* in both mitogenomes. In *P. resedaeformis* it is 2970 bp and in *A. digitatum*, is 2943 bp and is the shortest *mutS* gene sequenced among octocorals. The shortest gene in the mitogenomes is *atp8* subunit, which is 216 bp long in both newly sequenced genomes and also in all sequenced octocorals up to date. Gene length variation is most apparent in *nd2* and *mutS* genes while other gene sequences remain more stable.

Codon usage is almost the same in both species with ATG as a start codon and TAA or TAG as a stop codon. The only protein coding sequence where stop codons are not the same is *cob* gene. Here, TAA is the stop codon in *A. digitatum* and TAG in *P. resedaeformis*. Codon usage in all other protein-coding genes is the same in both genomes (Table 5).

Overall genome organization is highly compact in both species - all genes are involved in oxidative phosphorylation (except *mutS* which origin and function is still under the discussion) and are essential for performing key biochemical processes in the mitochondrion. A comparison of nucleotide sequences in coding regions revealed surprising level of similarity between *A. digitatum* and *P. resedaeformis* and a significant correlation with those of octocorals. Gene order matches Ancestral class (Table 9).

Table 9. Gene order classes in Octocorallia. The studied species are not followed by accession numbers.

Name	Classification	Size, nt	Geneorder class	Accession number
Alcyonium digitatum	Alcyonacea;Alcyoniina; Alcyoniidae;Alcyonium	18790	Ancestral	
Primnoa resedaeformis	Alcyonacea;Calcaxonia;Primnoidae;Primnoa	18726	Ancestral	
Acanella eburnea	Alcyonacea; Calcaxonia; Isididae; Acanella	18616	Keratoisidinae	NC_011016
Antillogorgia bipinnata	Alcyonacea; Holaxonia; Gorgoniidae; Antillogorgia	18733	Ancestral	NC_008157
Briareum asbestinum	Alcyonacea; Scleraxonia; Briareidae; Briareum	18632	Ancestral	NC_008073
Corallium elatius	Alcyonacea; Scleraxonia; Coralliidae; Corallium	18969	Konojoi	NC_022804
Corallium konojoi	Alcyonacea; Scleraxonia; Coralliidae; Corallium	18969	Konojoi	NC_015406
Corallium rubrum	Alcyonacea; Scleraxonia; Coralliidae; Corallium	18915	Konojoi	NC_022864
Dendronephthya castanea	Alcyonacea; Alcyoniina; Nephtheidae; Dendronephthya	18907	Ancestral	NC_023343
Dendronephthya gigantea	Alcyonacea; Alcyoniina; Nephtheidae; Dendronephthya	18842	Ancestral	NC_013573
Dendronephthya mollis	Alcyonacea; Alcyoniina; Nephtheidae; Dendronephthya	18844	Ancestral	NC_020456
Dendronephthya suensoni	Alcyonacea; Alcyoniina; Nephtheidae; Dendronephthya	18851	Ancestral	NC_022809
Echinogorgia complexa	Alcyonacea; Holaxonia; Paramuriceidae; Echinogorgia	19445	Ancestral	NC_020457
Euplexaura crassa	Alcyonacea; Holaxonia; Plexauridae; Euplexaura	18647	Ancestral	NC_020458
Junceella fragilis	Alcyonacea; Calcaxonia; Ellisellidae; Junceella	18724	Ancestral	NC_024181
Keratoisidinae sp. BAL 208-1	Alcyonacea; Calcaxonia; Isididae; unclassified Isididae	18923	Keratoisidinae	NC_010764
Narella hawaiinensis	Alcyonacea; Calcaxonia; Primnoidae; Narella	18838	Ancestral	NC_026192
Paracorallium japonicum	Alcyonacea; Scleraxonia; Coralliidae; Paracorallium	18913	Japonicum	NC_015405
Paraminabea aldersladei	Alcyonacea; Alcyoniina; Alcyoniidae; Paraminabea	19886	Ancestral	NC_018790
Scleronephthya gracillimum	Alcyonacea; Alcyoniina; Nephtheidae; Scleronephthya	18950	Ancestral	NC_023344
Sibagogorgia cauliflora	Alcyonacea; Scleraxonia; Paragorgiidae; Sibagogorgia	19030	Return to ancestral	NC_026193
Sinularia peculiaris	Alcyonacea;Alcyoniina; Alcyoniidae;Sinularia	18742	Ancestral	NC_018379
Heiopora coerulea	Helioporacea; Helioporidae; Heliopora	18957	Ancestral	NC_020375
Renilla muelleri	Pennatulacea; Sessiliflorae; Renillidae; Renilla	18643	Ancestral	NC_018378
Stylatula elongata	Pennatulacea; Subselliflorae; Virgulariidae; Stylatula	18733	Ancestral	NC_018380

<u>IGR structure</u>

Most intergenic regions (IGRs) show length and sequence similarity between *A. digitatum* and *P. resedaeformis*, but still several interesting features are noted.

First, *igr-1* is zero nucleotides in length which appears as highly conserved feature among the octocorals and shared by both *A. digitatum* and *P. resedaeformis.* Another example of resemblance in the intergenic region structure is *igr-10*. This region is in fact negative in both genomes (13 nt overlap between 3'-end of *nd2* and 5'-end of *nd5* genes) and octocorals studied.

Then, the largest IGR is *igr-11*, separate *nd5* and *nd4*. In *A. digitatum* it is 239 bp and in *P. resedaeformis* it consists of only 97 bp.

Mitotranscriptome

Mito - transcriptome sequencing gave different output for *A. digitatum* and *P. resedaeformis*. This may be because of different kits for rRNA depletion were used. Sequencing of *P. resedaeformis* resulted in low coverage (48 times and less) but a lot of transcripts of different genes gave nearly whole-mitogenome coverage. Most mapped transcripts correspond to ribosomal RNA subunits and *cox* genes (Fig.13). Some partial transcripts of *cob*, *nd2* and *2* subunits are also present though these are fragmented and scarce.

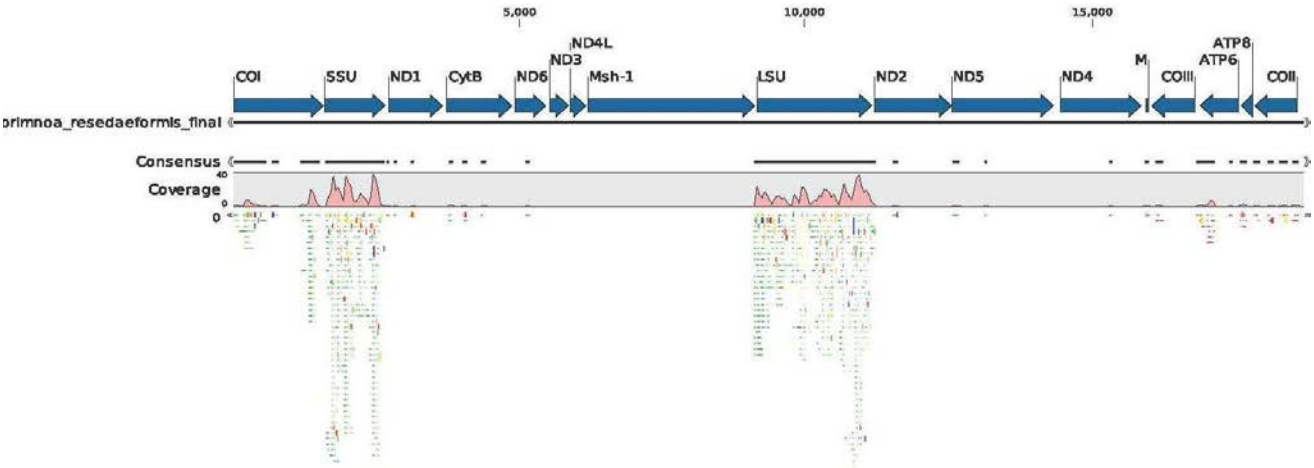


Figure 13. Transcriptome sequence mapping of the *P. resedaeformis* mitogenome

Sequencing of the *A. digitatum* mito-transcriptome resulted in 130 times coverage. *rns*, *rnl* genes and some parts of *cox1* and *nd5* map the most of the transcripts (Fig. 14).

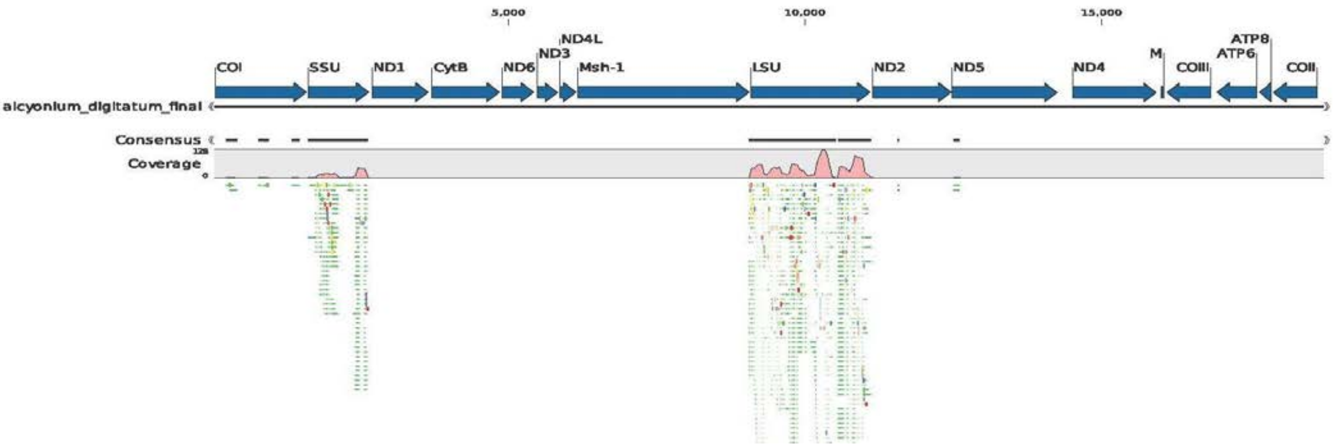


Figure 14. Transcriptome sequence mapping of *A. digitatum* mitogenome

Molecular cloning

Sequencing, assembly and annotation revealed a region of high variability. Though the gene sequence has no frame shifts, it was subjected to molecular cloning procedures with the intention of amplifying by genetically transformed *E.coli* and further verification by Sanger sequencing.

Transformation reaction was successfully performed and resulted in white colonies of genetically modified *E.coli*. Insertion of *mutS* gene was experimentally proved by PCR. Sanger sequenced fragments are in the range of 700 - 1000 bp. Mapping of fragments (see Fig. 15) on corresponding genomes gives strong support for Ion Torrent sequenced mitogenomes and this highly variable region, particularly.

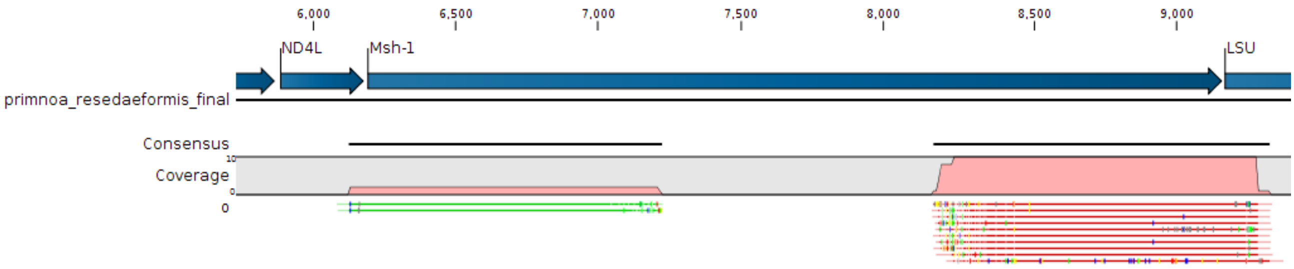


Figure 15. An example of mapping of cloned and Sanger sequenced fragments of mutS gene on the mitogenome of *P. resedaeformis.* Products cover 5'-end and 3'-end of the gene sequence.

Phylogenetic analysis

1. Dataset 1: concatenated multiple alignments of *mutS* gene
2. Dataset 2: concatenated multiple alignments of re-annotated protein-coding sequence

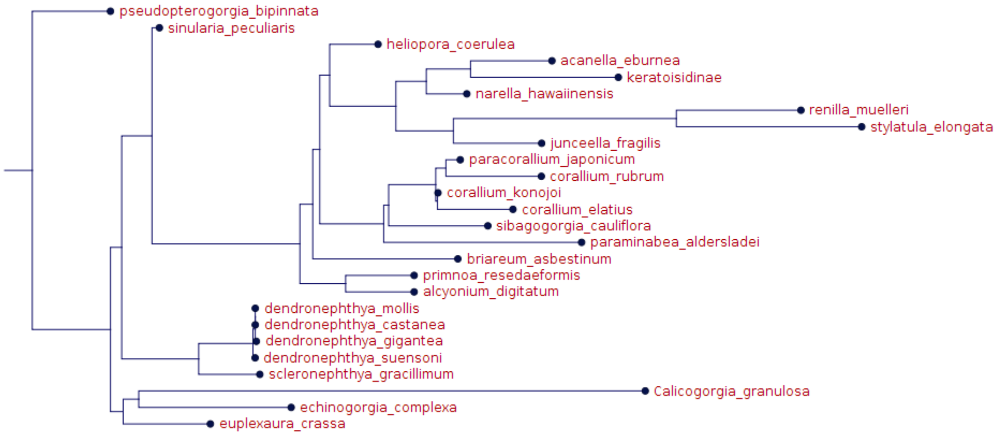


Figure 16. Phylogenetic tree based on dataset 1: distance between *mutS* gene sequences in octocorals.

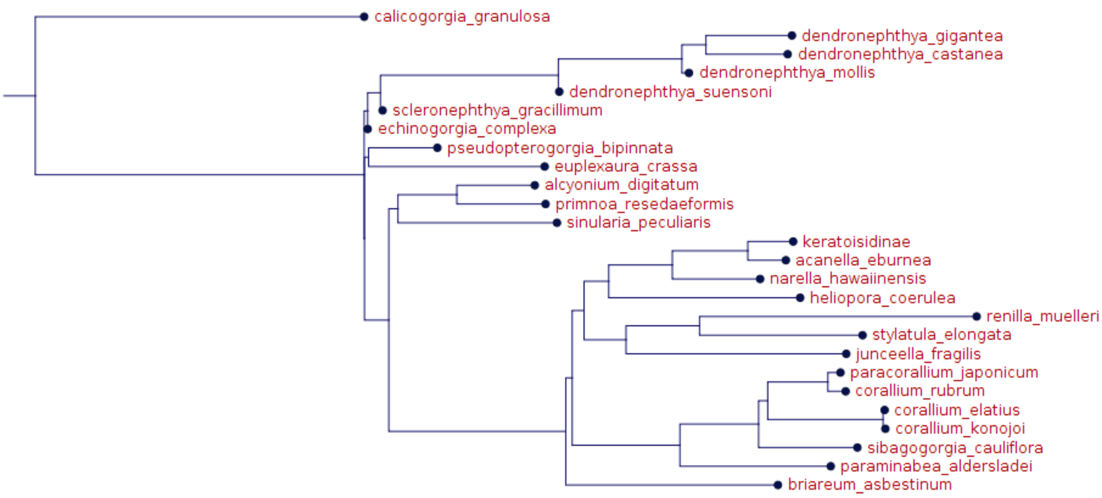


Figure 17. Phylogenetic tree based on dataset 2: distance between re-annotated protein coding sequences in octocorals.

As can be seen from the Figure 16, distance in the nucleotide sequence of *mutS* gene between *P. resedaeformis* and *A. digitatum* is small, suggesting that these species are related.

Figure 17 represents strong support for previous tree, where the studied species are grouped together. Tree topology is apparently similar, but branch lengths are changed.

Discussion

Octocoral mitogenomes

Two new mitogenomes of the octocoral species *A. digitatum* and *P. resedaeformis* were assembled and annotated based on the sequenced data produced by the current study. They were found to correspond closely in size, gene arrangement and gene sequences with previously sequenced octocorals. We explored sequence features of the two novel mitogenomes in comparison with other available octocoral mitogenome sequences.

We found a relatively low level of nucleotide variations among octocoral species in most of protein coding genes and rRNA genes. Such scant variability is in the line with several findings of recent studies. Most studies agree there is a result of a general tendency for mitogenome size reduction within Metazoa (Bernt et al., 2013; Osigus et al., 2013), economically organized mitogenomes are especially pronounced in Octocorallia.

Another plausible reason for the low level of variability could be the presence of an active *mutS* gene. The *MutS* gene seems to be a specific and obligatory gene for all octocoral mitochondrial genomes. Actively expressed *mutS* gene seems to prevent mutations by DNA mismatch repair, which would slow down evolutionary rates of the mitogenome in general. Based on the complete octocoral mitogenome sequences published so far, *mutS* seems to be actively expressed in all species.

Both origin and function of this gene are still under the discussion. However, in a comprehensive study researchers compared amino acid sequences of octocoral, bacterial and viral *mutS*-family proteins. As a result, horizontal gene transfer and non-eukaryotic origin of this gene was proposed. Functional gene product was strongly suggested by the authors due to the presence of all required domains and a deduced protein structure that indicates involvement in mismatch repair (Bilewitch and Degnan, 2011).

Paradoxically, this gene is also the most variable within the class harboring more nucleotide and amino acid variation that the remaining protein coding genes among octocorals. Thus, the *mutS* sequence has been shown to evolve faster than other parts of mitogenome of octocorals (France and Hoover, 2001; van der Ham et al., 2009). Strong positive selection for continued presence of *mtMuts* gene in the mitogenome deduced as an explanation of this phenomenon (Bilewitch and Degnan, 2011).

In the studied species, *mutS* gene also possess notable variability. The sequence of this gene in *A. digitatum* is almost the shortest within the class and differs significantly from the corresponding region in *P. resedaeformis*.

Another variable part of the mitogenomes is the *nd2* gene sequence. As it was presented in the alignments, this region is also an example of outstanding variability expressed in large 5'-heterogeneous part of the gene. It is also obvious from the alignment that the studied species are close in this sequence to more distantly related species - *H. coerulea, S. elongata* and *R. muelleri*. The protein alignment reflects these differences. The rest of the gene sequence is recognizable within the set of octocoral species.

These variable gene sequences are interesting topic for future research. Here, a gene product variability and structure can be studied within the class. Sequencing of several species of *Alcyonium* and *Primnoa* can be applied for studying gene sequence mobility and protein structure variability and function within the family.

Furthemore, our finding is the shortest *cox1* genes that also possess incomplete termination codon T. It was documented in most species from Alcyonacea family in this region and seems to be common in these organisms (*cox1* octocoral papers). Incomplete termination codon indicates posttranscriptional modifications such as polyA restore. Unfortunately, no transcript were sequenced from this region of the sequence. Therefore, it is a feature to be determined in further investigations.

In addition to previous findings, IGR structure and gene sequences are very similar in Alcyonacea family and remain relatively conserved within the class. Total sizes presented in the Results are within the common range (414 to 957 bp) of intergenic spacers for octocorals (McFadden et al., 2010). An interesting feature observed in the studied mitogenomes is the *igr-11* of *A. digitatum*. This *igr* is among the most variable regions in the intergenic space of octocoral mitochondrion. In *A. digitatum* demonstrates a distinguished size that exceeds the length of *igr-11* in closely related species twice. Curiously, this region in *P. resedaeformis* does not show such deviation from congeneric species.

An interesting feature which could be detected here is a presence of repeated sequences that are pervasive across Metazoa mitogenomes (Nardi et al., 2012). They are believed to contribute to frequent appearance of mutation. Author, however, mentions, that a tendency to possess repeated sequences is met in groups with more flexible mitogenome lengths. Thus, they could be expected in the mitogenomes of Hexacorallia rather than Octocorallia. Test of repeated sequences was not carried out, but described feature of *A. digitatum* is interesting to investigate thoroughly.

It would also be useful to explore *igr* content closely for the purpose of detection a replication origin. Though replication origin is not well documented in Cnidarians (Bernt et al., 2013), it is believed to reside in the *igr-17* that separates *cox2* and *cox1* genes (Uda et al., 2011), where authors found a hairpin-forming sequence. Therefore, screening for secondary structure can lead to detection of this region.

Gene overlaps represent another remarkable feature of octocoral intergenic architecture and are believed to prevent rearrangement events (Brugler and France, 2008). Indeed, it was observed in *igr-10* that can serve as an example of such features. This region is highly consistent within octocoral mitogenomes and "holds" *nd2* and *nd5* genes together by 13 nucleotides overlap. As a result, *nd2* and *nd5* genes are placed together in all mitogenomes within octocorals despite of gene order class. It is reflected in the overall mitogenome organisation where rearrangements do not change this region.

Evolutionary rates of octocoral genomes are suggested to be 5 times slower than rates of nuclear genome (Chen et al., 2009). Furthermore, when it comes to comparison with another groups of animals, a 50-100 times slower rates of mtDNA in octocorals are suggested (Hellberg, 2006). Lower evolutionary rates of mitochondrial protein coding genes are equilibrated by higher tendencies for gene rearrangements in the mitogenome (Uda et al., 2011). These rearrangements reflect evolutionary history of this phylum which is expressed in gene segments shuffling and duplication (Figueroa and Baco, 2014; Park et al., 2012). Given events are believed to appear at several times, with ancestral class as an initial state and *konojoi* and *japonicum* classes as variations of those. A new trend - return to ancestral state - was also described in mitogenome of *S. cauliflora*. Our investigations allow to define sequenced mitogenomes as matching Ancestral gene order class (Figueroa and Baco, 2014).

Nucleotide sequence alignment of both ribosomal RNA subunits show expected level of similarity. However, as presented in the alignments, sequences still possess regions with gaps and dissimilarities. Nucleotide variations are abundant in these genes, and this information can be successfully used for phylogenetic reconstructions. The use of rRNA genes in phylogenetic analyses is discussed in the next chapter.

Octocorallia. tRNA retention is documented within Cnidaria (Beagley et al., 1995; Flot et al., 2008). Within Octocorallia only tRNA fMet complement is presented in mitogenomes and obligatory for all representatives. This transfer RNA contains formyl-methionine - a start-codon that initiates translation of protein-coding genes. Other tRNAs are imported in the mitochondrion space by cytosolic transportation.

Mitotranscriptome sequencing revealed actively expressed genes. Genes with high coverage are validating annotation. However, coverage and quality of reads are moderate. This can be caused by kits used for the library preparation. More specific and sensitive isolation protocols and kits can be suggested for successful extraction of non-degraded RNA and library preparation process.

In the end, our data is in the line with previous knowledge and main tendencies of taxa of interest, although curious details revealed. There are also several suggestions that can improve further studies.

Mitochondria enrichment protocols can be proposed for more successful output from mtDNA extraction procedures. Dense sampling could also help in creation of representative dataset. Here, sampling for suborder and family representatives will highlight the reason of deviation from common patterns. Moreover, this strategy would allow detailed phylogenetic analyses and improve currently unresolved taxa. Particularly, sampling and mitogenome sequencing of species from Helioporacea, Pennatulacea and other groups will increase the resolution of analyses. Sampling of specimens of *A. digitatum* and *P. resedaeformis* would promote detecting of SNPs, selective sweeps, haplotype diversity and population structure characteristics.

Phylogenetic assays

Anthozoan subclasses have diverged 500 million years ago and are clearly separated in phylogenetic trees (Kayal et al., 2013). No intermediate groups between Octocorallia and Hexacorallia documented. Application of NGS revealed differences in the mitogenome content of this subclasses (Emblem et al., 2014). However, some nodes still remain unresolved in cnidarian tree of life and, particularly in octocorals (Park et al., 2011). Anthozoan phylogeny is an example of such group and its origin has also been questioned since mitogenome content became uncovered.

Paraphyly of this group was suggested by (Park et al., 2012). This study exploits large data set of protein coding genes and concludes that Octocorallia is rather a sister taxa to Medusozoa and Hexacorallia is likely to be divergent (Kayal et al., 2013). Osigus et al. (2013) also suggest Octocorallia to be a sister group to monophyletic Medusozoa and Hexacorallia grouping outside or together with sponges (Demospongiae and Homoscleromorpha). These recent studies exploit comprehensive datasets based on protein-coding sequeces of all (or nearly all) genes. This approach is beneficial.

Some studies suggest phylogenetic inferences based on one or several genes (Morris et al., 2012). However, this strategy is not successful. As was discussed earlier, octocoral mitogenomes are very special in their invariability. Because of these specific features of octocoral mitogenomes, it is not completely correct to use single gene sequence or even a combination of them, as was shown by McFadden et al. (2011). "Octocoral barcoding" based on c*ox1+mtMutS+igr1* was suggested by the authors in the attempt of applying barcoding approach in this group of animals. A combination of genes was used since the *cox1* gene sequence is insufficiently variable in octocorals, and the *mutS* gene sequence is too variable, oppositely. This approach would be suitable in the absence of complete mitogenome sequences, but it would not reflect the whole set of relationships between species. Since more molecular data become available, the use of phylogeny inferences based solely on one (or few) gene sequence become incorrect.

Re-annotated and consequently concatenated mitogenomes are advantageous for the use in revealing phylogenetic relationships (Kayal et al., 2013). Although, higher levels of phylogenetic require an addition of rRNA genes and, in some cases, sequences of intergenic regions.

Nuclear genes are also used in the phylogenetic reconstructions. In Sánchez et al. (2003) 18S rRNA was added to mitochondrial 16S rRNA coding sequence. Different branching patterns were observed in the resulting trees, though topology was recognizable in general. This could be a result of an influence of different rates of sequence evolution in either genomes, as was discussed earlier. Despite in the given study phylogenetic analysis is based on a single gene sequence, usefulness of combining nuclear and mitochondrial gene sequences was shown. Involvement of nuclear genes would facilitate the phylogenetic analyses, but requires nuclear gene sequences and experience in interpretation of phylogenetic analyses.

In our analyses, *A. digitatum* and *P. resedaeformis* were used in the phylogenetic analyses and are grouped together in both phylogenetic trees. High bootstrap value gives strong support for the results. It is a remarkable result since species belong to the two different families – Alcyoniina and Calcaxonia. It was suggested in Sánchez et al. (2003), these families could be polyphyletic, based on 16S sequences. However, if more coding sequences were used, the resulted phylogenetic analysis would be more robust. A good strategy for resolving phylogenetic uncertainties is to involve a combination of protein coding genes, ribosomal RNA subunits sequences, and intergenic regions. This can be an interesting topic for further studies.

Moreover, if more sequenced mitogenomes of Calcaxonia were available, it would significantly improve phylogenetic tree resolution. Therefore, further studies are crucially important for adequate conclusions.

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Appendix A

Nucleic acid extraction protocols

DNA extraction protocols

Urea protocol

1) Add 500 µl tissue Tissue lysis buffer in MagNalyser tube

2) Add small piece of tissue (half a pea or smaller)

3) Homogenize

4) Add 20 µl (20 mg/ml) proteinase K

5) Incubate in 55ºC 1-2 hours.

6) Transfer homogenate to new Eppendorf tube

7) Add 500 µl of phenol/chloroform (basic pH), vortex well to mix

8) Spin 10000 rpm 10 min, transfer water phase to new tube

9) Do the extraction (add another portion of phenol-chloroform) again until the layer between the two phases is almost clear (first time it is often white and fat)

10) Extract one time with chloroform/isoamyl (same volume as the waterphase), vortex and spin

11) Transfer water phase again, precipitate the DNA in the water phase by adding 2,5x volume of 100% EtOH and 1/10 volume 3M NaAc, mix

12) Put in -20 ºC freezer for 1 hour

13) Spin down in cold centrifuge 13000 rpm 30 min.

14) Wash the pellet with 1 ml 70% EtOH (several times if much salt, pipet up-down, vortex, spin 10 min 4 ºC 13 rpm)

15) Rehydrate the pellet in 20 µl clean water or LowTE. Keep in -20 freezer

RNA extraction protocols

Standard TRIzol protocol, originally modified for cod, MG group ( )

1. 500-700 µl Trizol in MagNalyser tube

2. Add small piece of tissue (half a pea or smaller)

3. Homogenize until fully homogenized

4. Add 0,2 x volume of cold chloroform, incubate on ice for 20 min, shake occasionally

5. Centrifuge at 4ºC 20 min 9000 rpm

6. Transfer water phase to a new Eppendorf tube

7. Precipitate RNA by adding 1x volume of cold isopropanol, incubate at 4 ºC for 1 hour

8. Centrifuge at 13000 rpm for 30 min

9. Remove supernatant carefully, wash with 1 ml cold 80% EtOH, centrifuge 5 min at 13000 rpm.

10. Remove all EtOH with a pipette carefully

11. Leave to dry for a few minutes and resuspend in water (20 µl or wanted volume).

Appendix B

PCR primers

Table A1 contains primers used in this study. Primers were used for amplification in PCR and sequencing by Sanger method. Primers constructed by Aase Emblem, UiN. Forward primers named F and reverse primers named as R, respectively.

Name	Sequence (5'->3'), length	Vol for 100\µmol	GC-content, %	Use for PCR/ sequencing
Pre mutS_F	CTGCCATGAGTGGGCATAGTC(21)	233	57.1	P/S
Pre mutS_R	GACTATGCCCACTCATGGCAG (21)	302	57.1	P
Pre COI_F	GCAGTGGACATGGCCATATTCAG (23)	279	52.2	P/S
Pre COI_R	CTGAATATGGCCATGTCCACTGC (23)	288	52.2	P/S
Pre ND4_F	GGAGTTCTCACCTCAACTAG (20)	326	50	P/S
Pre ND4_R	CTAGTTGAGGTGAGAACTCC(20)	282	50	P/S
Pre ND4L_F	CTGTCGCAGCTGCCGAGTCTGC(22)	261	68.2	P/S
Pre ND4L_R	GCAGACTCGGCAGCTGCGACAG(22)	282	68.2	P/S
Pre CytB_F	GCTATCCCTTATGTAGGCACAG(22)	314	50	P/S
Pre CytB_R	CTGTGCCTACATAAGGGATAGC (22)	256	50	P
Pre ND1_F	GCCCGGGCATCGTACTAGCTG(21)	340	66.7	P
Pre ND1_R	CAGCTAGTACGATGCCCGGGC(21)	300	66.7	P
Pre CO2_F	GTCAGTGTTCCGAGCTATGTG(21)	342	52.4	P/S
Pre CO2_R	CACATAGCTCGGAACACTGAC(21)	247	52.4	P
Pre CO3_F	CAGTAACARGGGCACATCACGC(22)	292	54.5	P
Pre CO3_R	GCGTGATGTGCCCATGTTACTG(22)	352	54.5	P/S

Pre ND5_F	CTCCATAGCATCTGGCAACC(20)	258	55	P/S
Pre ND5_R	GGTTGCCAGATGCTATGGAG(20)	326	55	P/S
Pre ND2_F	GCGCTAAGATAGTAGCCCTG(20)	334	55	P
Pre ND2_R	CAGGGCTACTATCTTAGCGC(20)	294	55	P/S
Oct_SSU1_F	GACCTTGGGGAGTCTATAAG(20)	383	50	P/S
Oct_SSU1_R	CTTATAGACTCCCCAAGGTC(20)	328	50	P
Oct_SSU2_F	GGCAGCAGTAGAGAATCTTG(20)	368	50	P/S
Oct_SSU2_R	CAAGATTCTCTACTGCTGCC(20)	406	50	P
Oct_SSU3_F	GCATTAGGCGTTAAAGTATG(20)	442	40	P
Oct_SSU3_R	CATACTTTAACGCCTAATGC(20)	407	40	P/S
Oct_SSU4_F	CAAGTTAAGGATACGAGACGC(21)	255	47.6	P/S
Oct_SSU4_R	GCGTCTCGTATCCTTAACTTG(21)	421	47.6	P
Oct_Met_F	GAGTTGAACCTACATAACCAG(21)	372	42.9	P
Oct_Met_R	CTGGTTATGTAGGTTCAACTC(21)	367	42.9	P/S
Oct_LSU1_F	GAGATTCCGTACGTAGCGG(19)	342	57.9	P/S
Oct_LSU1_R	CCGCTACGTACGGAATCTC(19)	430	57.9	P
Oct_LSU2_F	CTTGGATGAGCTGTGGTTAGC(21)	313	52.4	P/S
Oct_LSU2_R	GCTAACCACAGCTCATCCAAG(21)	312	52.4	P/S
Oct_LSU3_F	CACTGAATGAGCTAGGAAACC(21)	328	47.6	P/S
Oct_LSU3_R	GGTTTCCTAGCTCATTCAGTG(21)	348	47.6	P
Oct_LSU4_F	CGTAATCAACTTCTGGCTGCTGC(23)	350	52.2	P/S
Oct_LSU4_R	GCAGCAGCCAGAAGTTGATTACG (23)	341	52.2	P/S
Pri_ND2_F	ACCGTTACTAAGTGCAGTCGG(21)	386	52.4	P
Pri_ND2_R	TCCGACTGCACTTAGTAACGG(21)	371	52.4	P/S
Pri_ND2b_F	AGGCAGATGGATAGAGTCCG(20)	381	55	P/S
Pri_ND2b_R	TCGGACTCTATCCATCTG(20)	387	55	P/S

Successful primer combinations

Table A2 contains successful combinations of PCR primers and lists the reactions whose products were subjected to Sanger sequencing.

Species	Primer F	Primer R	Sanger sequenced product
P. resedaeformis	PremutSF	PreND2R	DNA+PreND2R
P. resedaeformis	mutS F	PreND4LR	DNA+PreND4LR
P. resedaeformis	CytBF	PreND4LR	DNA+PreND4LR
P. resedaeformis	PreCOI F	PreND5R	DNA+PreND5R
P. resedaeformis	PreCOIF	PreND4LR	DNA+PreND4LR
P. resedaeformis	PreCO2F	PreCOIR	DNA+PreCOIR
P. resedaeformis	PreCOII F	Pre COIII R	
P. resedaeformis	PreND4F	PreND4L F	DNA+PreND4L F
P. resedaeformis	PreND5F	PreCO III R	
P. resedaeformis	PreND4LF	PreND2R	
P. resedaeformis	PreND4LF	PreND4R	
P. resedaeformis	LSU1 F	LSU4R	DNA+LSU1F, DNA+LSU4R
P. resedaeformis	LSU3F	PriND2bR	DNA+LSU3F
P. resedaeformis	SSU1 F	LSU4R	DNA+SSU1 F
P. resedaeformis	LSU4F	MetR	
P. resedaeformis	SSU2F	MetR	DNA+SSU2F
P. resedaeformis	SSU4F	SSU3R	DNA+SSU4F, DNA+SSU3R
P. resedaeformis	PriND2bF	LSU3R	
P. resedaeformis	SSU1F	LSU4R	
A. digitatum	OctLSU2F	OctND2R	DNA+OctLSU2F, DNA+OctND2R

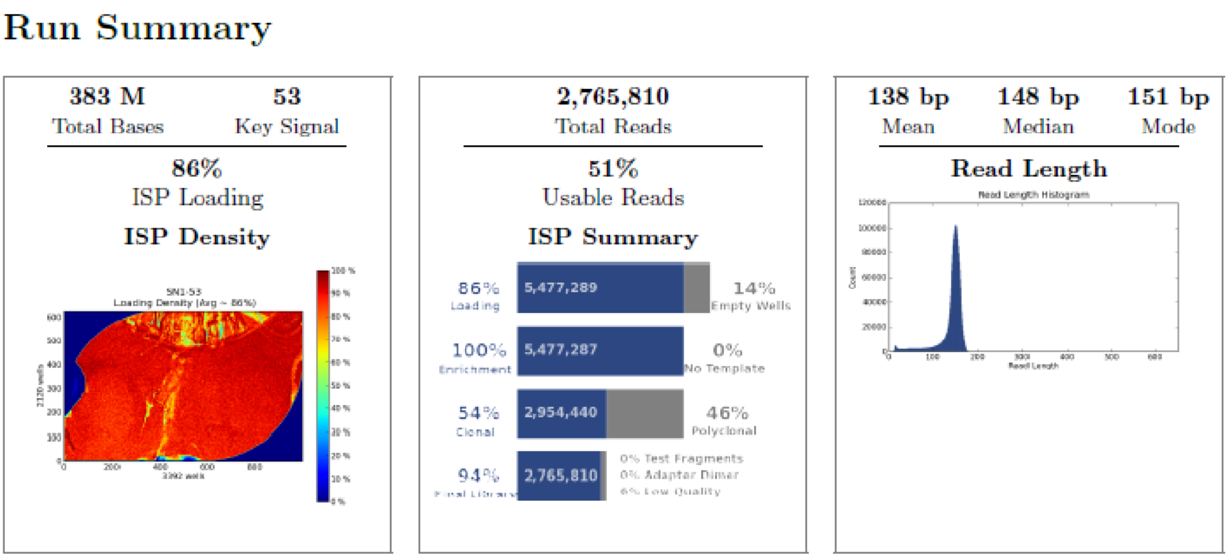
PCR primers used for cloning

Table A3 includes primers used for molecular cloning. Primers are based on the IonTorrent assembled mitogenome regions. They were constructed by Å. Emblem and ordered from Eurofins Genomics.

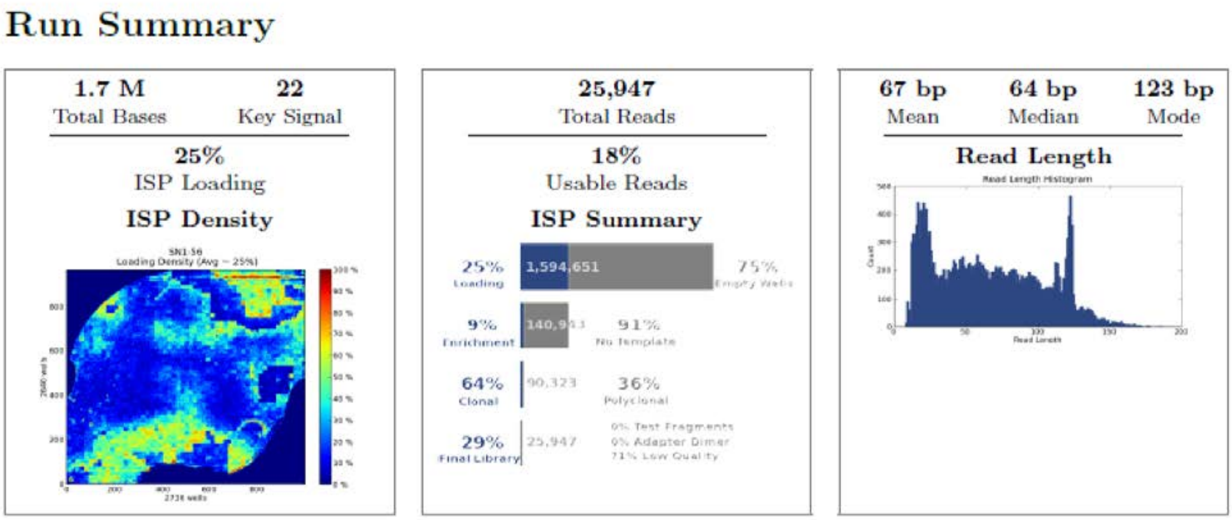
Name	Sequence (5’->3’), length	Vol for 100\µmol	GC-content, %	Use for PCR/ cloning
Pr_MutS_F	ACGTGGTACAATTGCTGTTC(21)	308	45	P/C
Pr_MutS_F2	CTGTCGCAGCTGCCGAGTCTG(21)	317	66.7	P/C
Pr_MutS_R	TCCTCATAGTCTAGTAAGATG(21)	312	38.1	P/C
Pr_MutS_R2	CGCTATCGCTCGCCGCT(21)	303	71.4	P/C

Appendix C

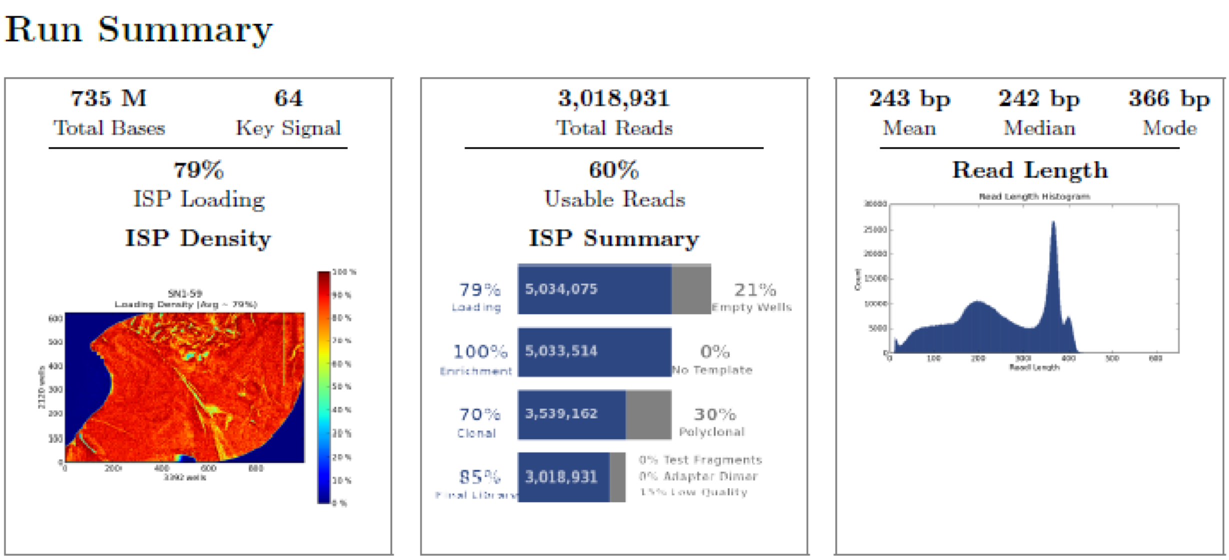
Sequencing run summary on *P. resedaeformis* DNA



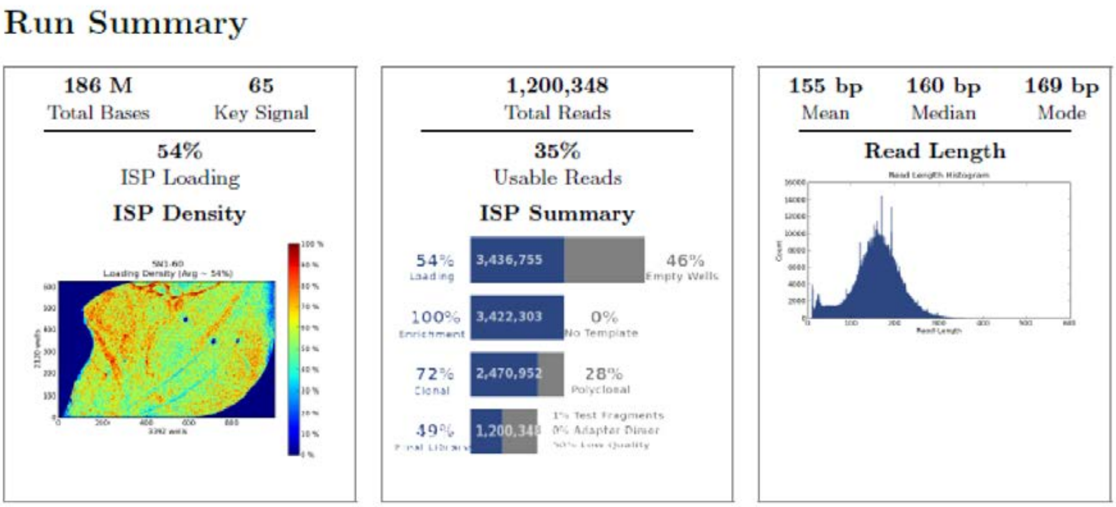
Sequencing run summary on *P. resedaeformis* cDNA



Sequencing run summary on *A.digitatum* DNA



Sequencing run summary on *A.digitatum* cDNA



Appendix D

Alcyonium digitatum **mitogenome sequence**

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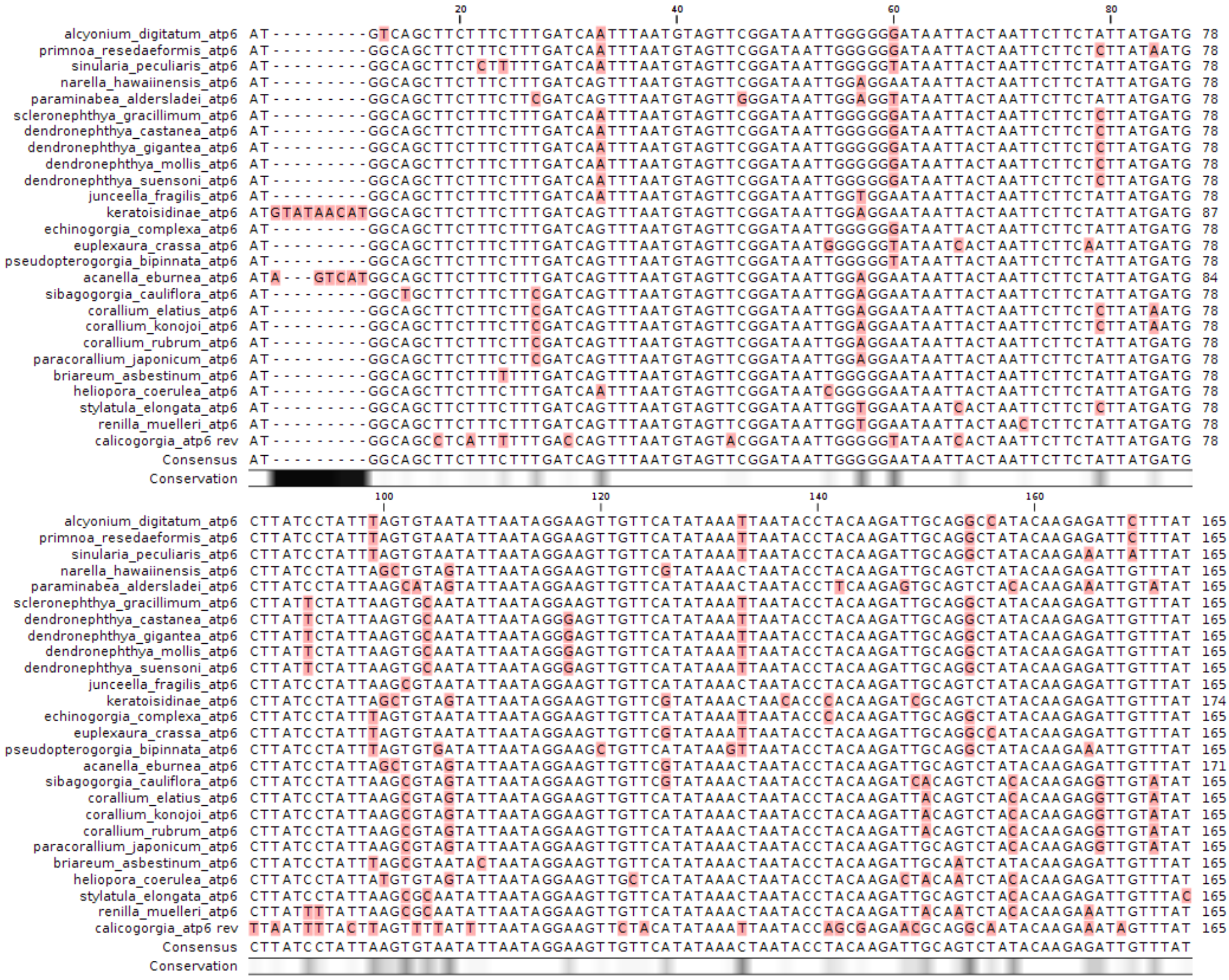
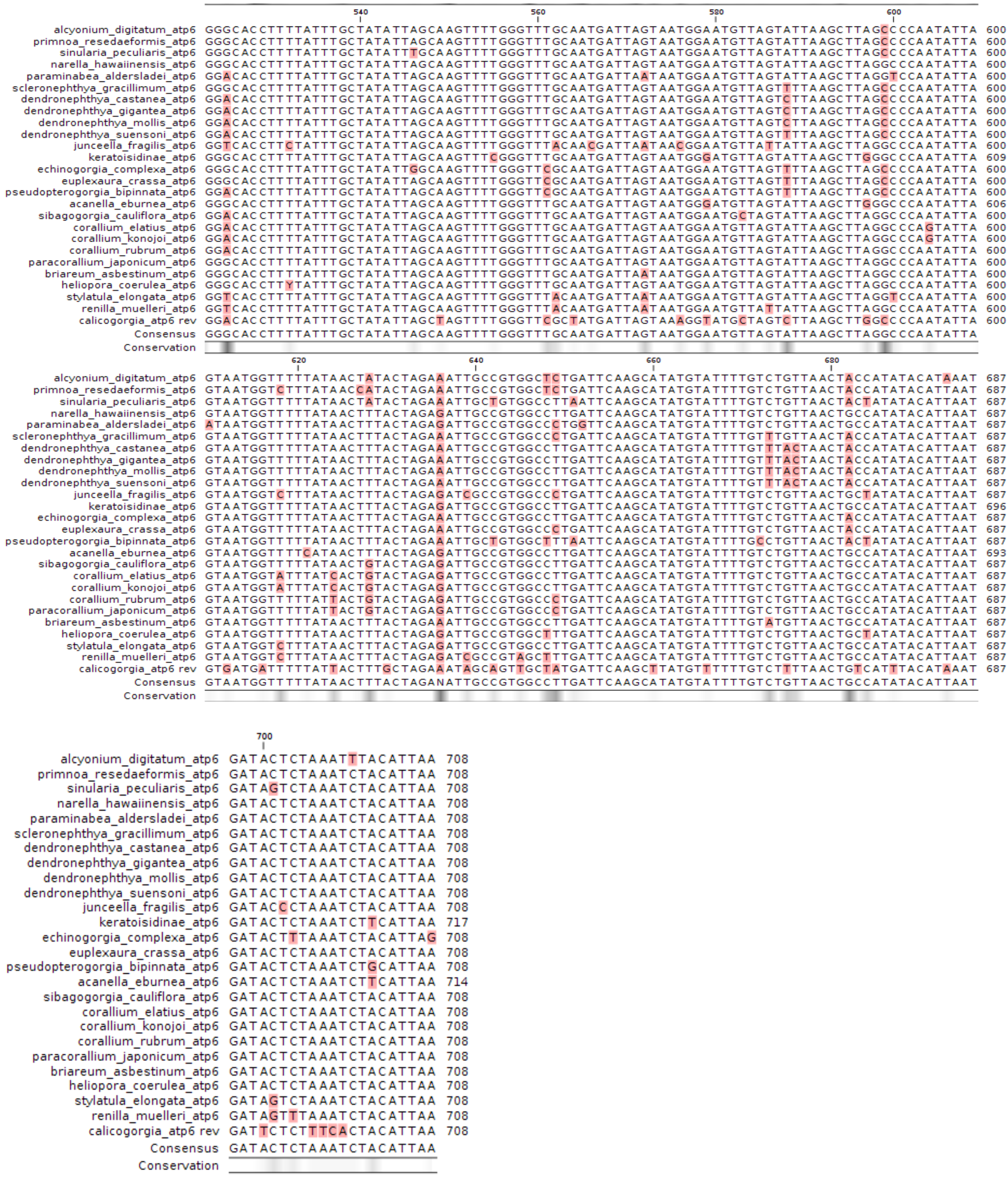
Appendix E

Multiple nucleotide alignments of protein coding and rRNA genes of *A. digitatum*, *P. resedaeformis* and all available complete mitochondrial genome

***Atp6* gene**





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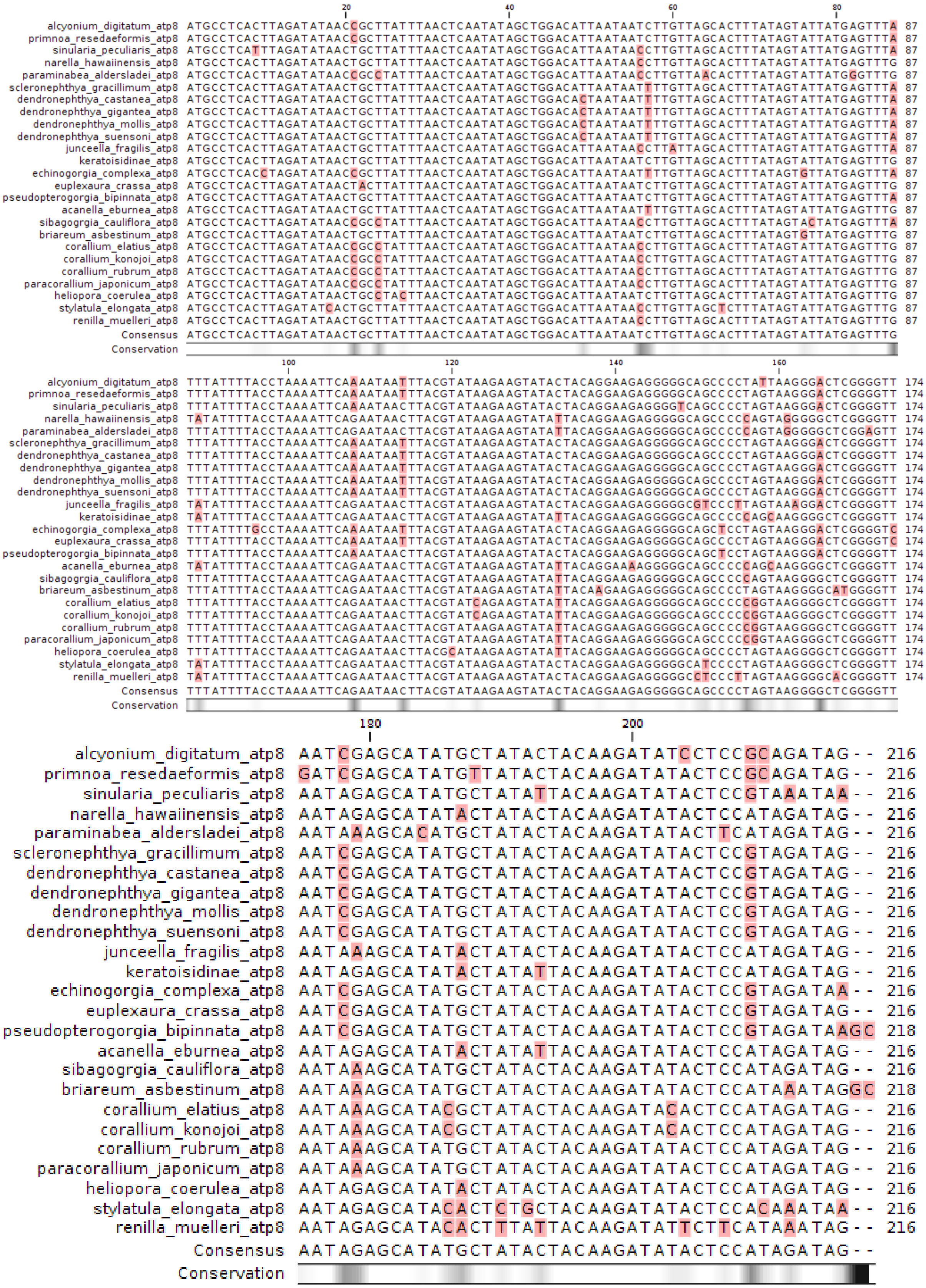
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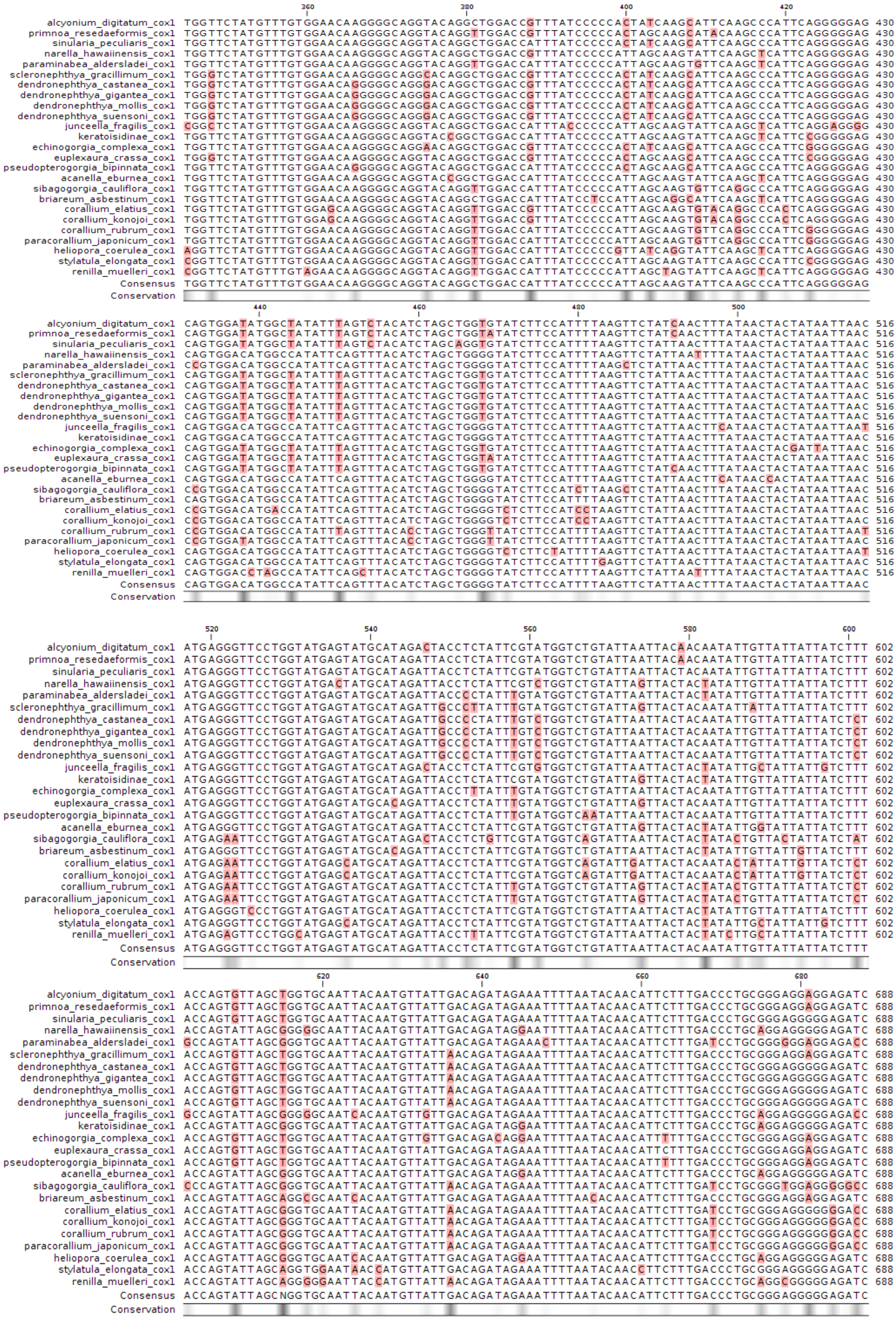
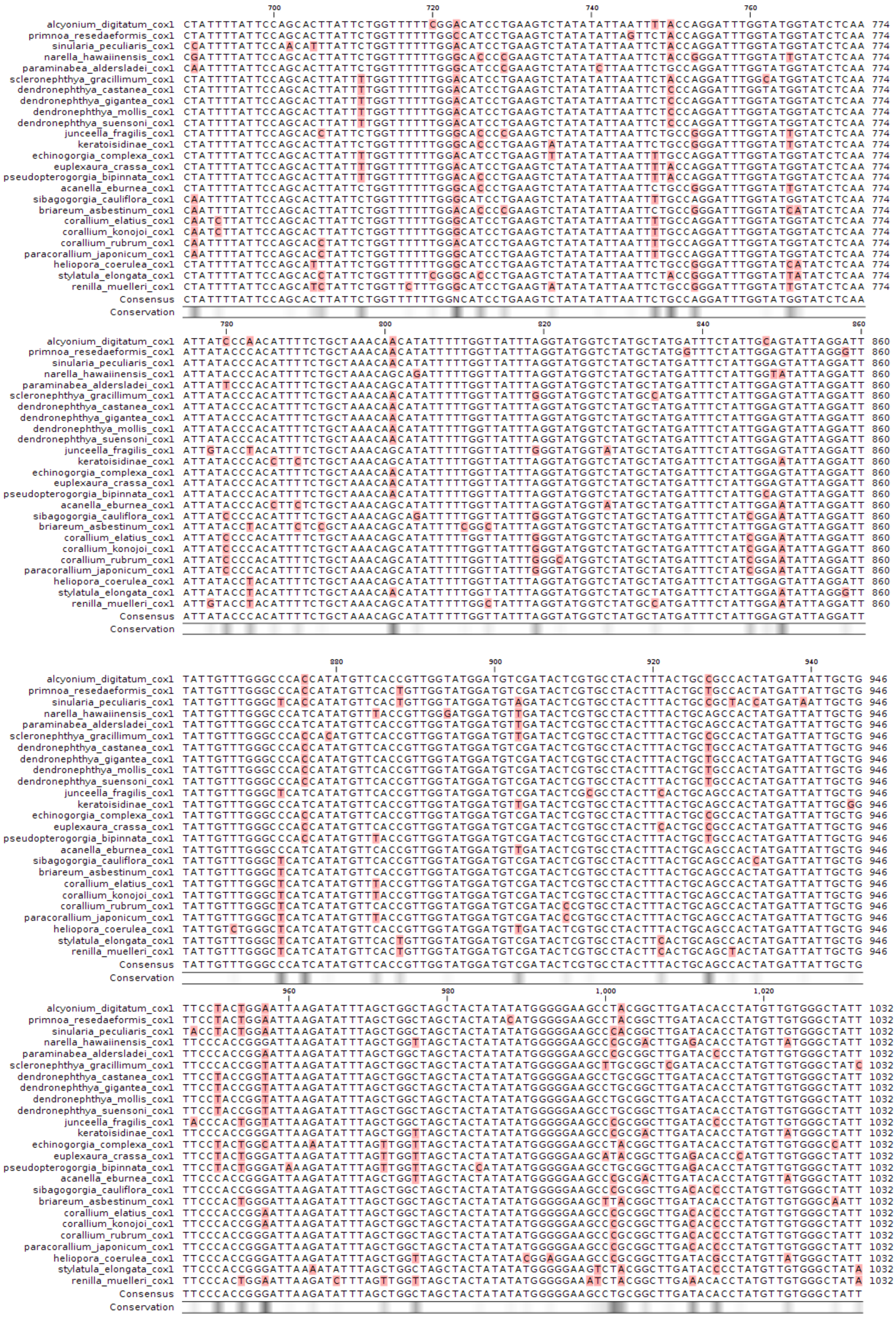
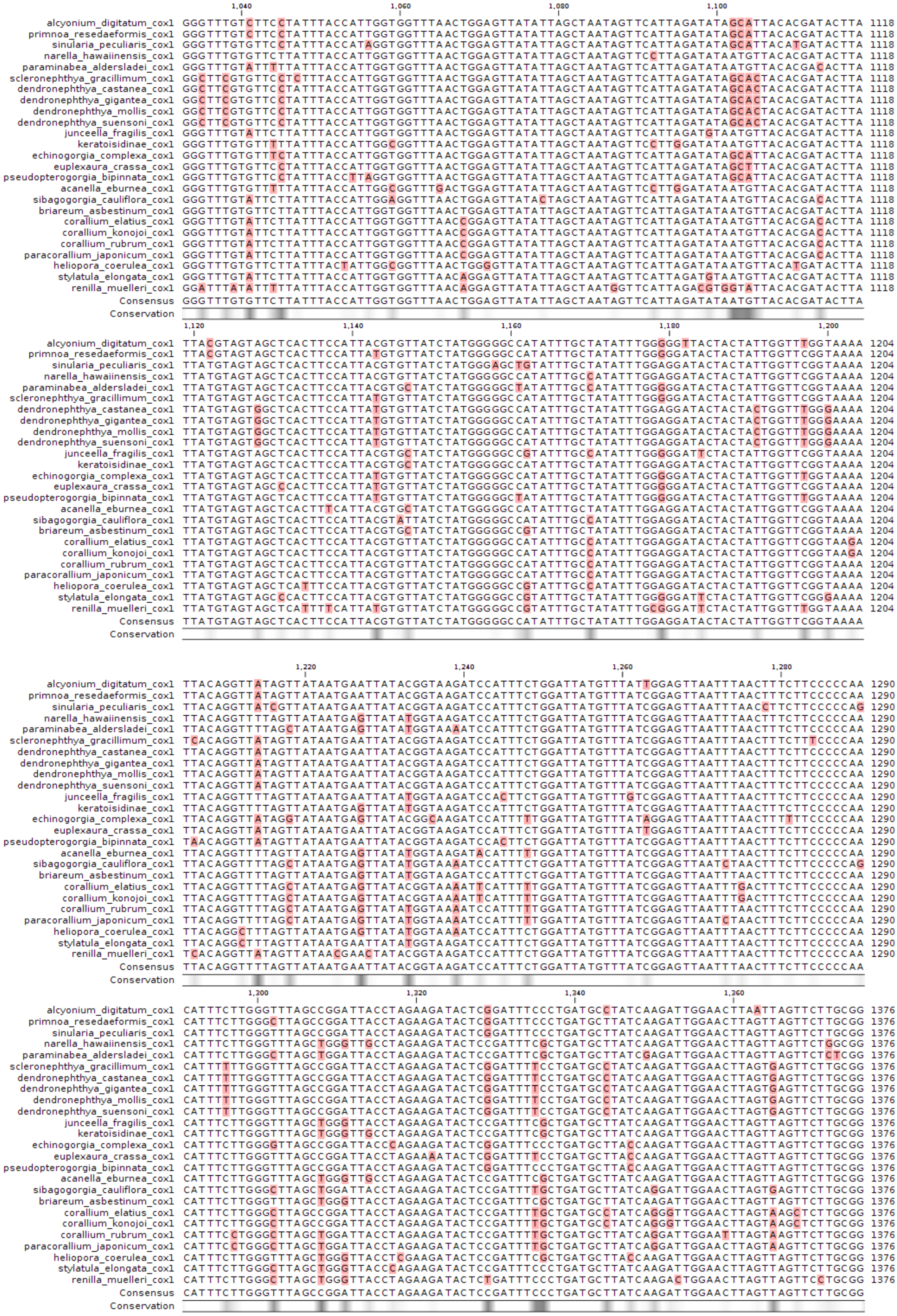
Consensus
Conservation

Cox1 gene



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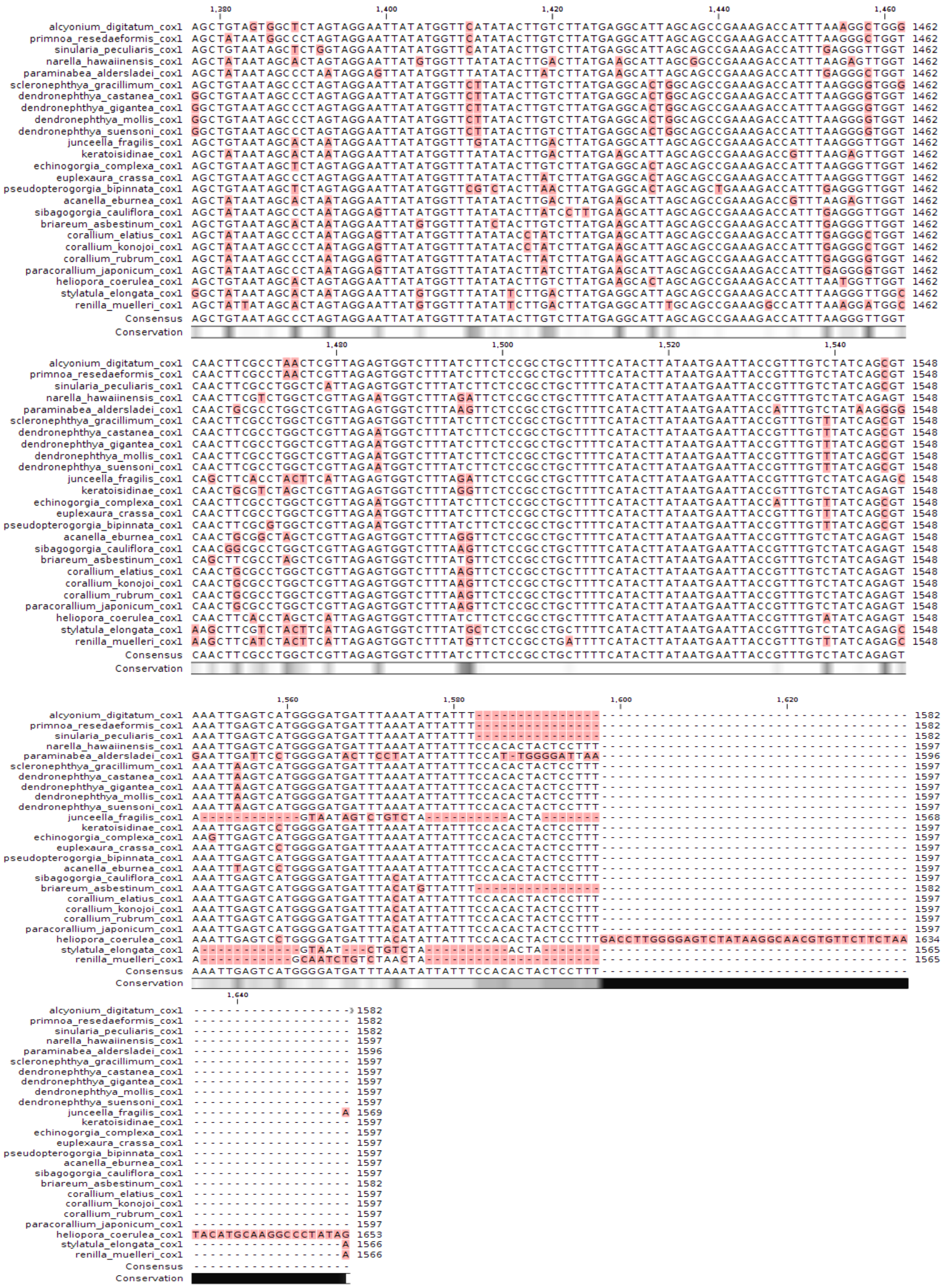
Conservation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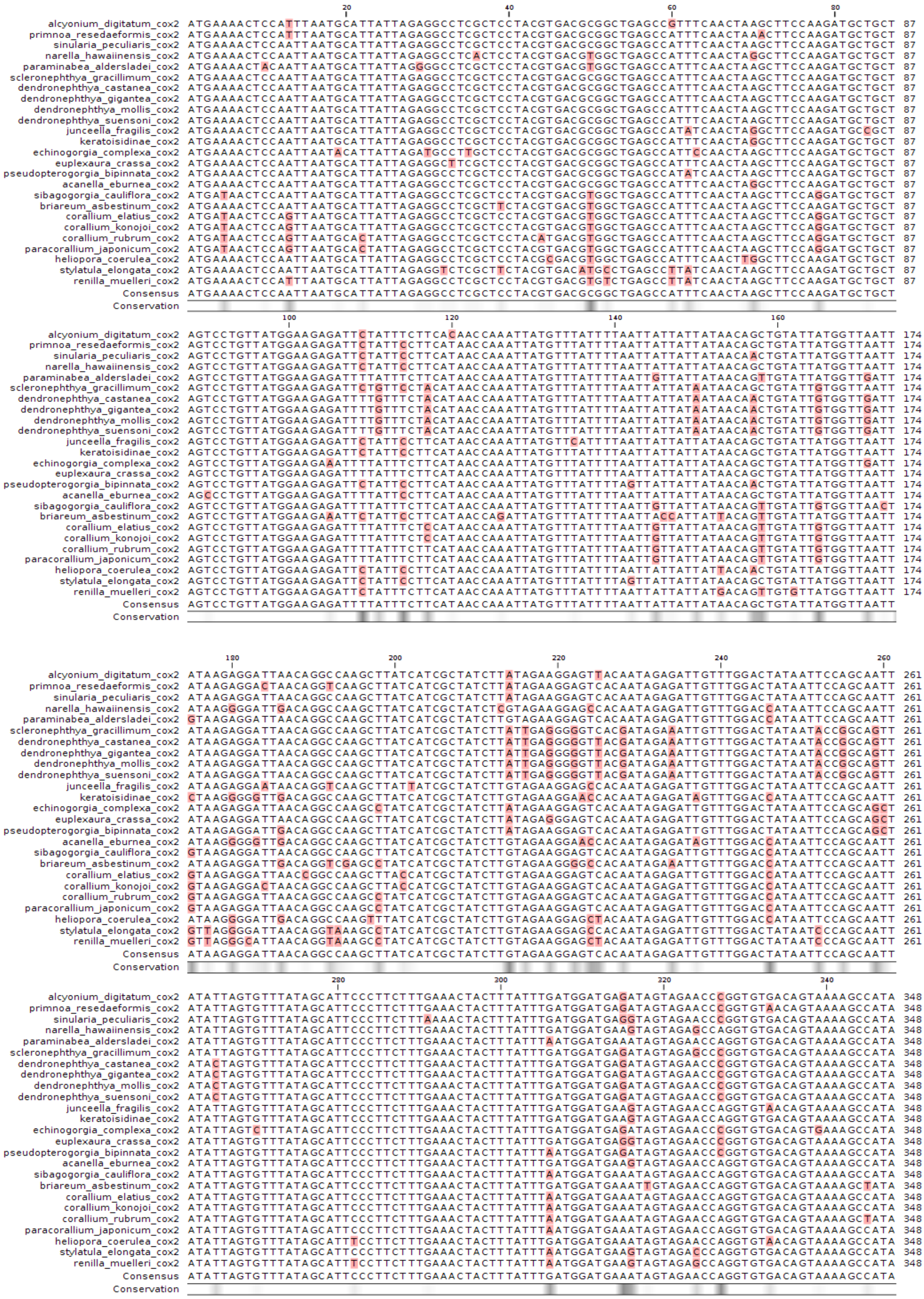
Consensus
Conservation

Cox2 gene



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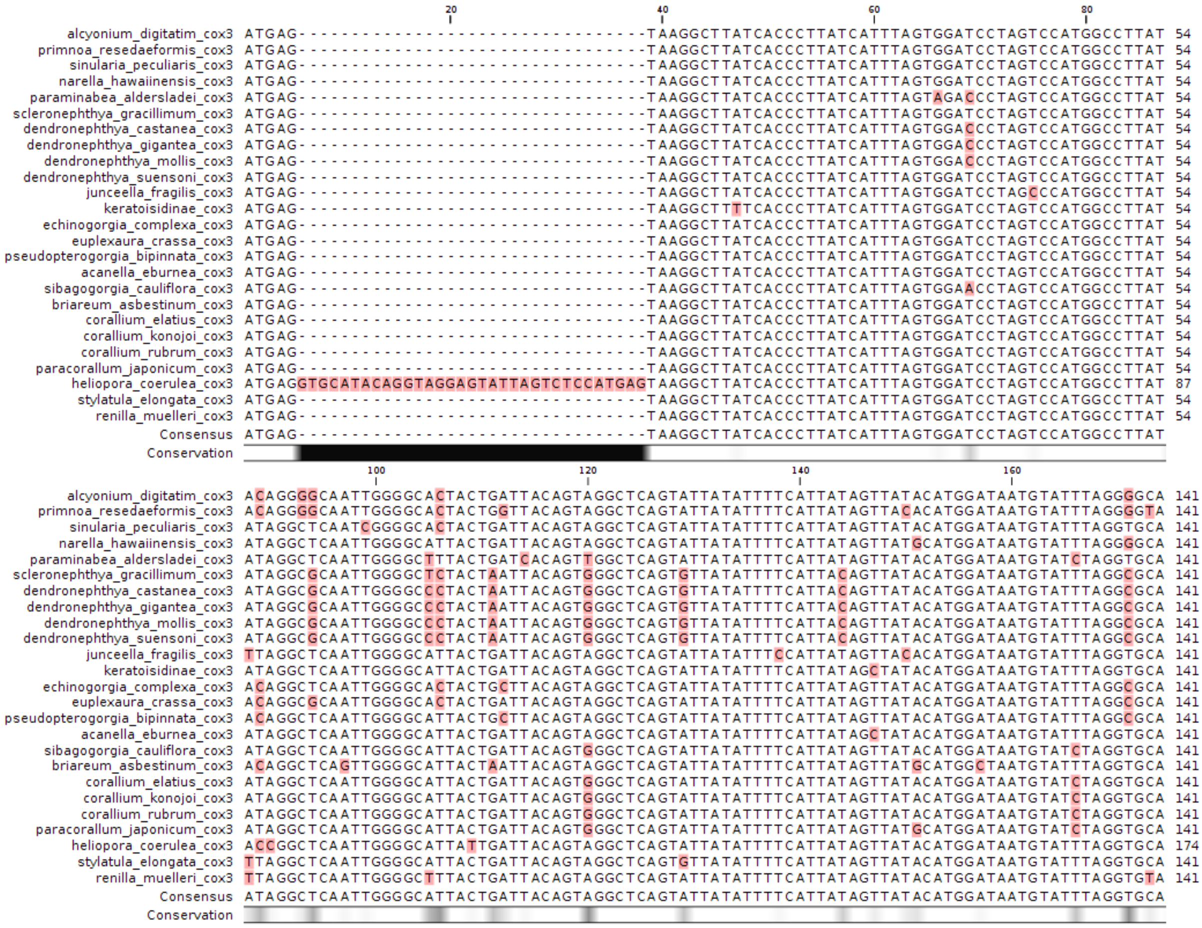

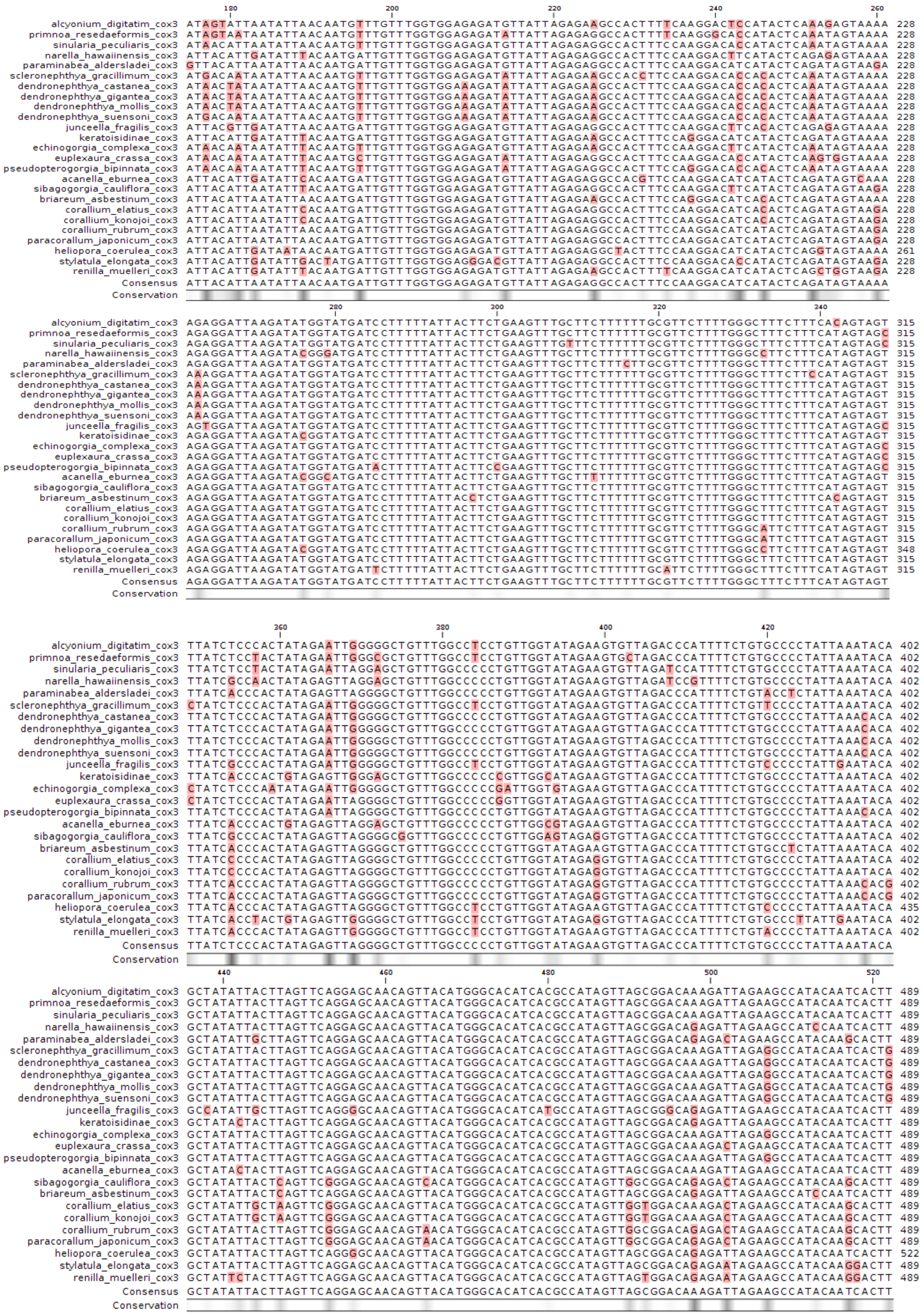
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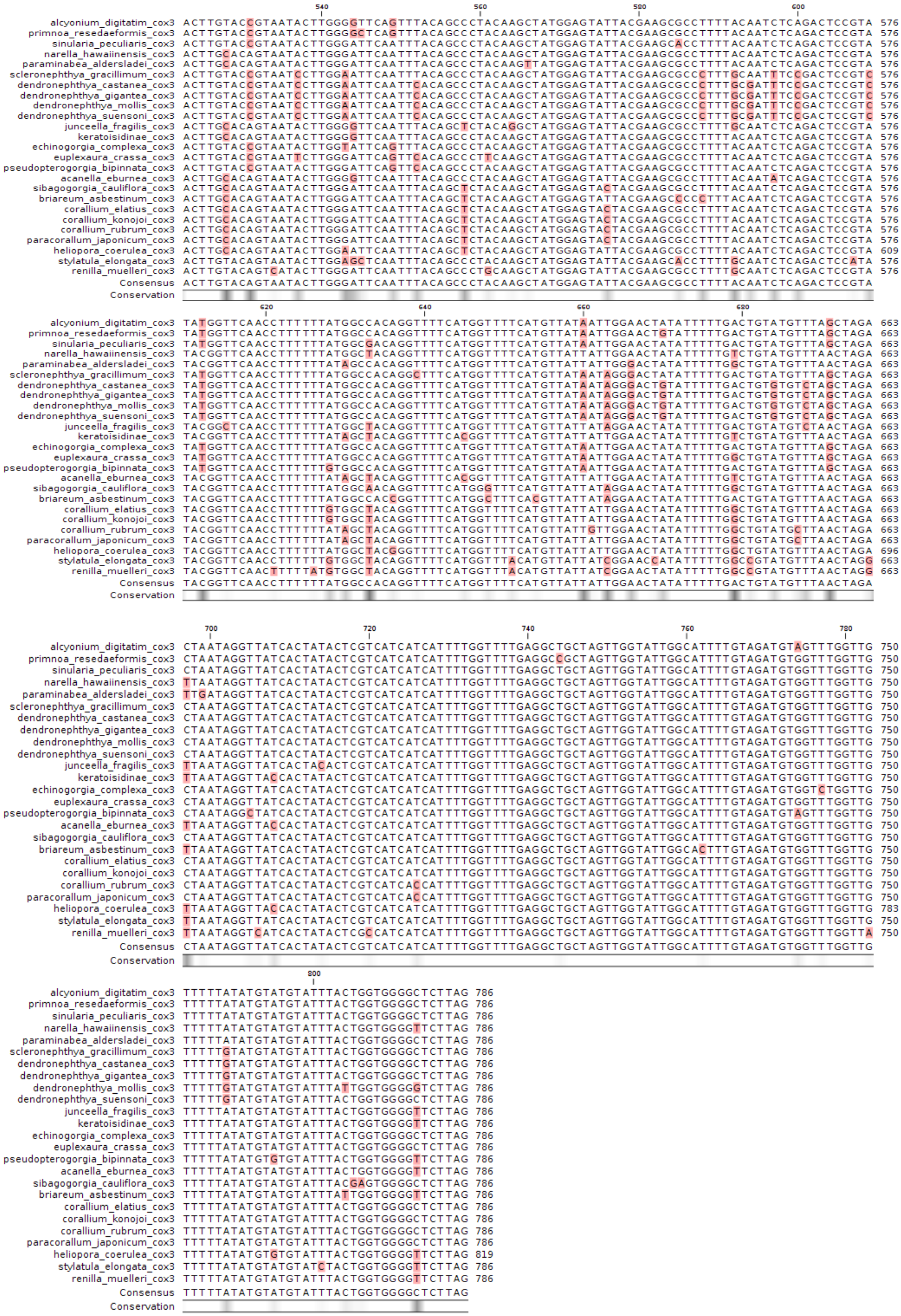

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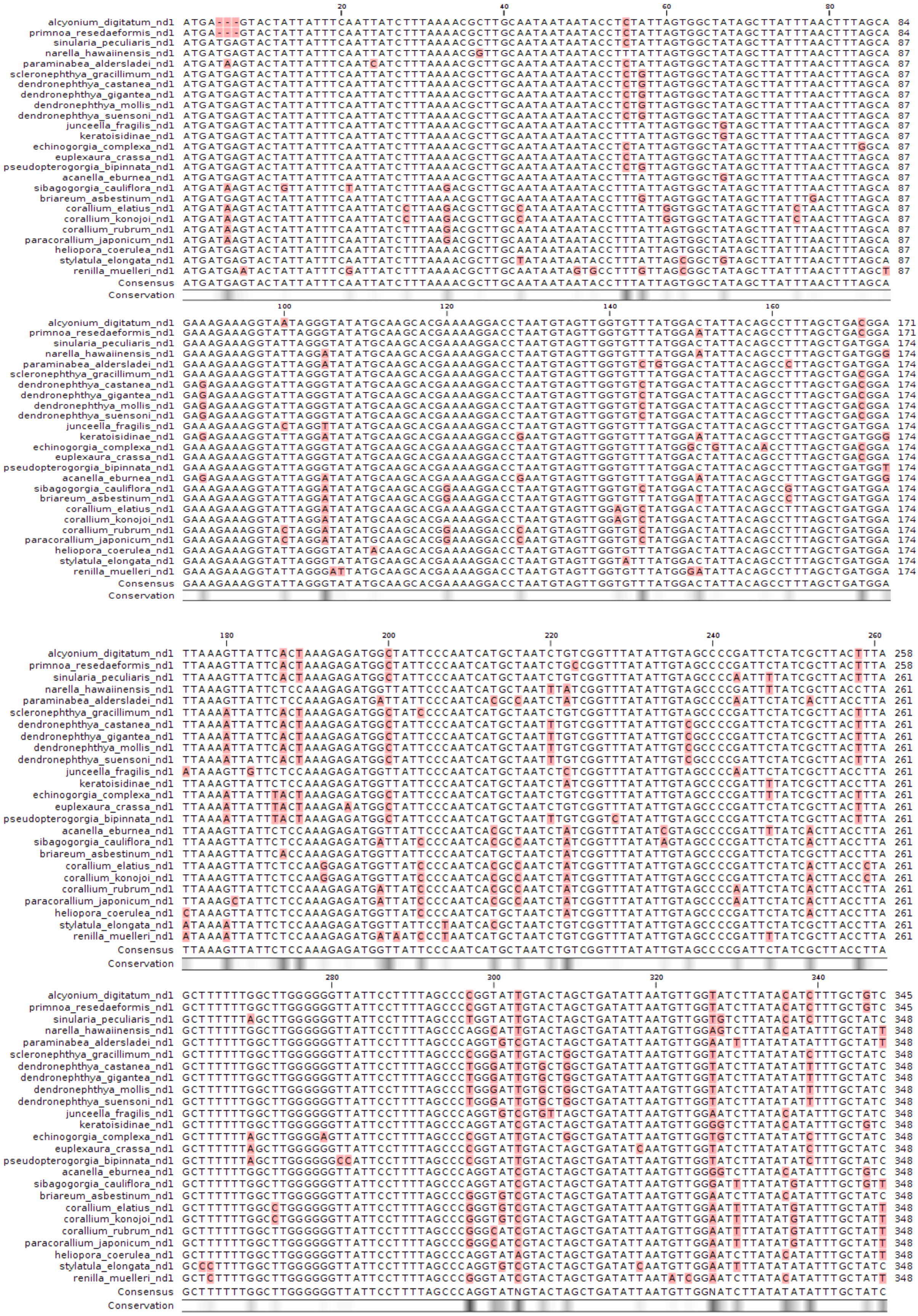

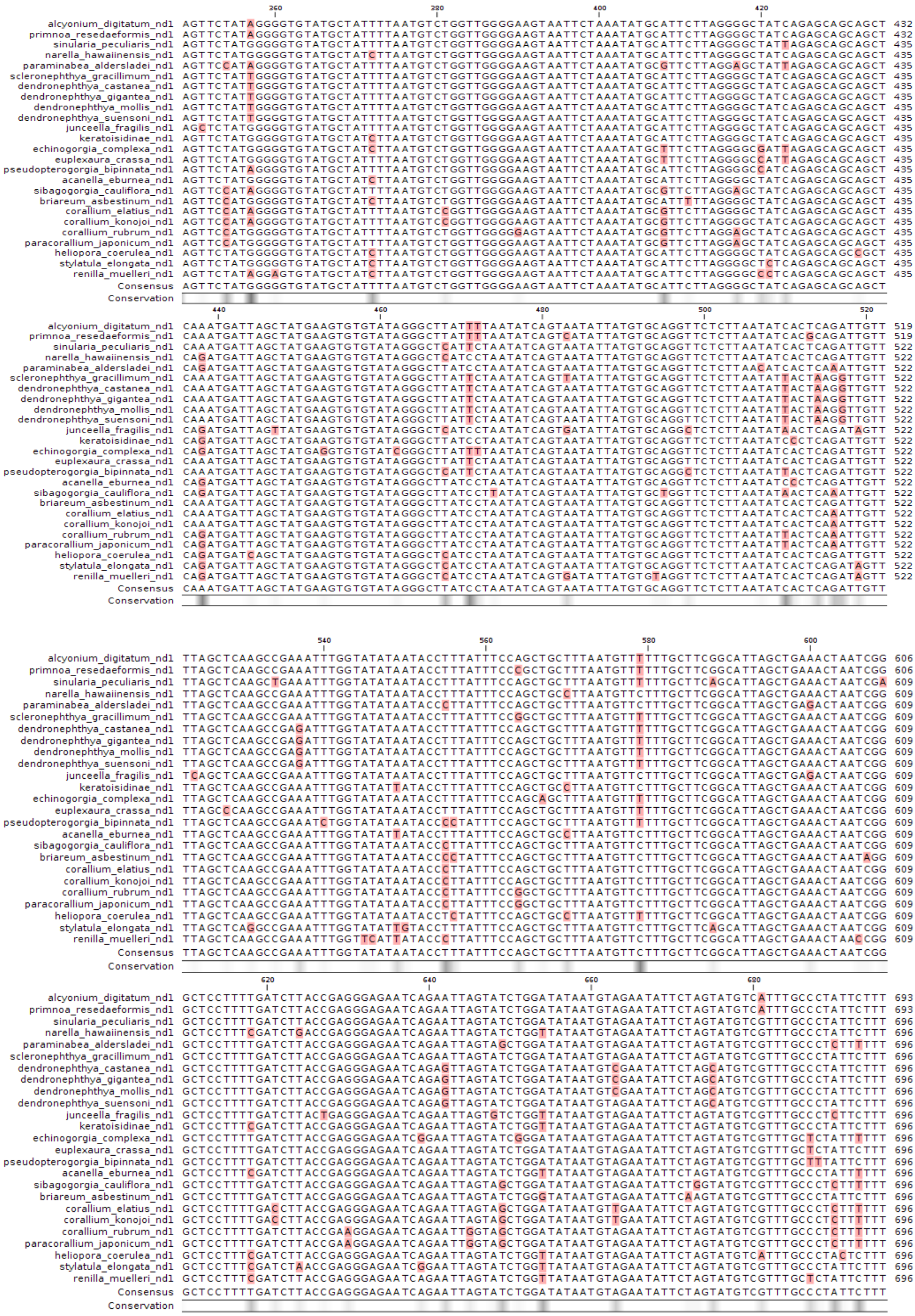

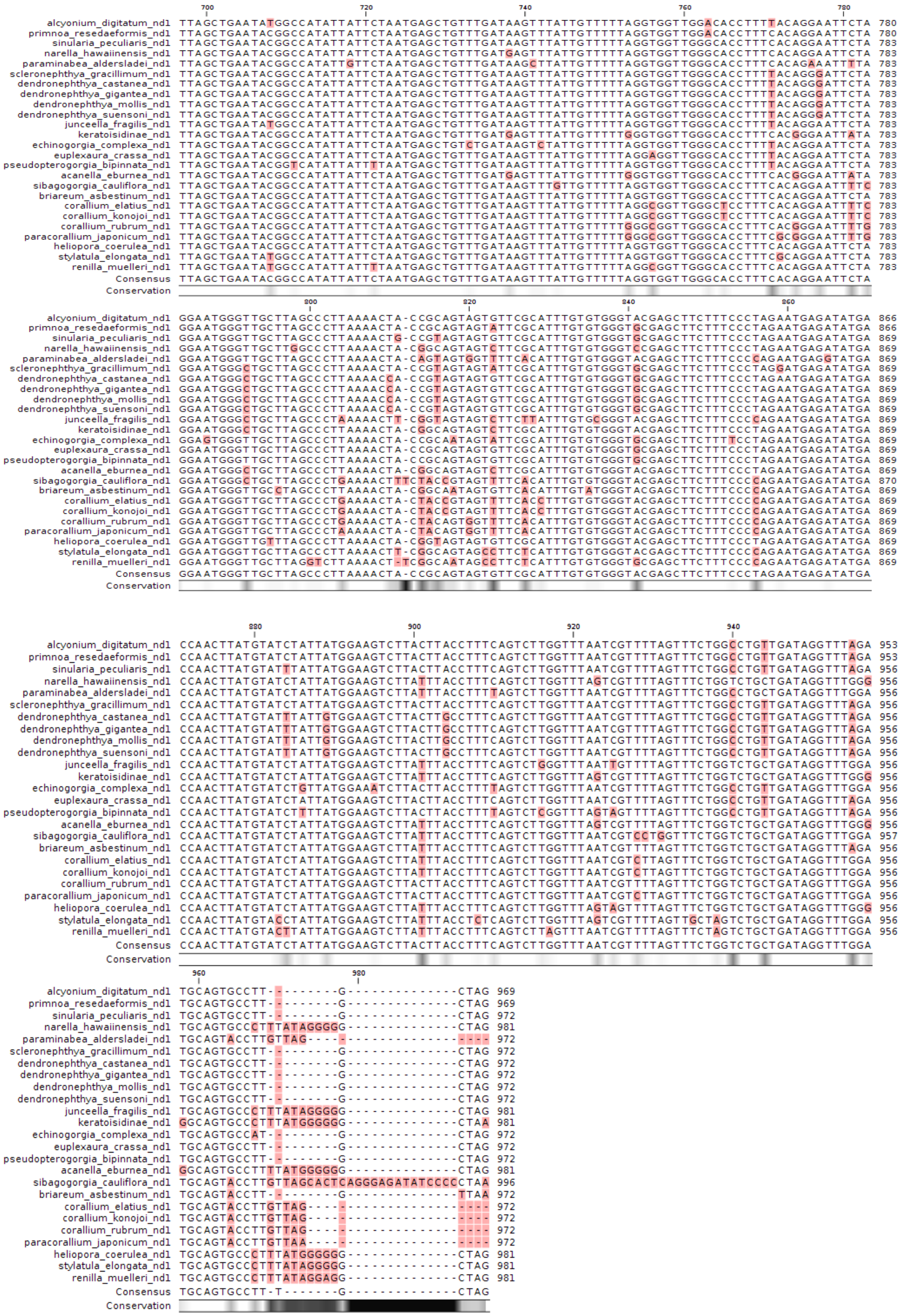

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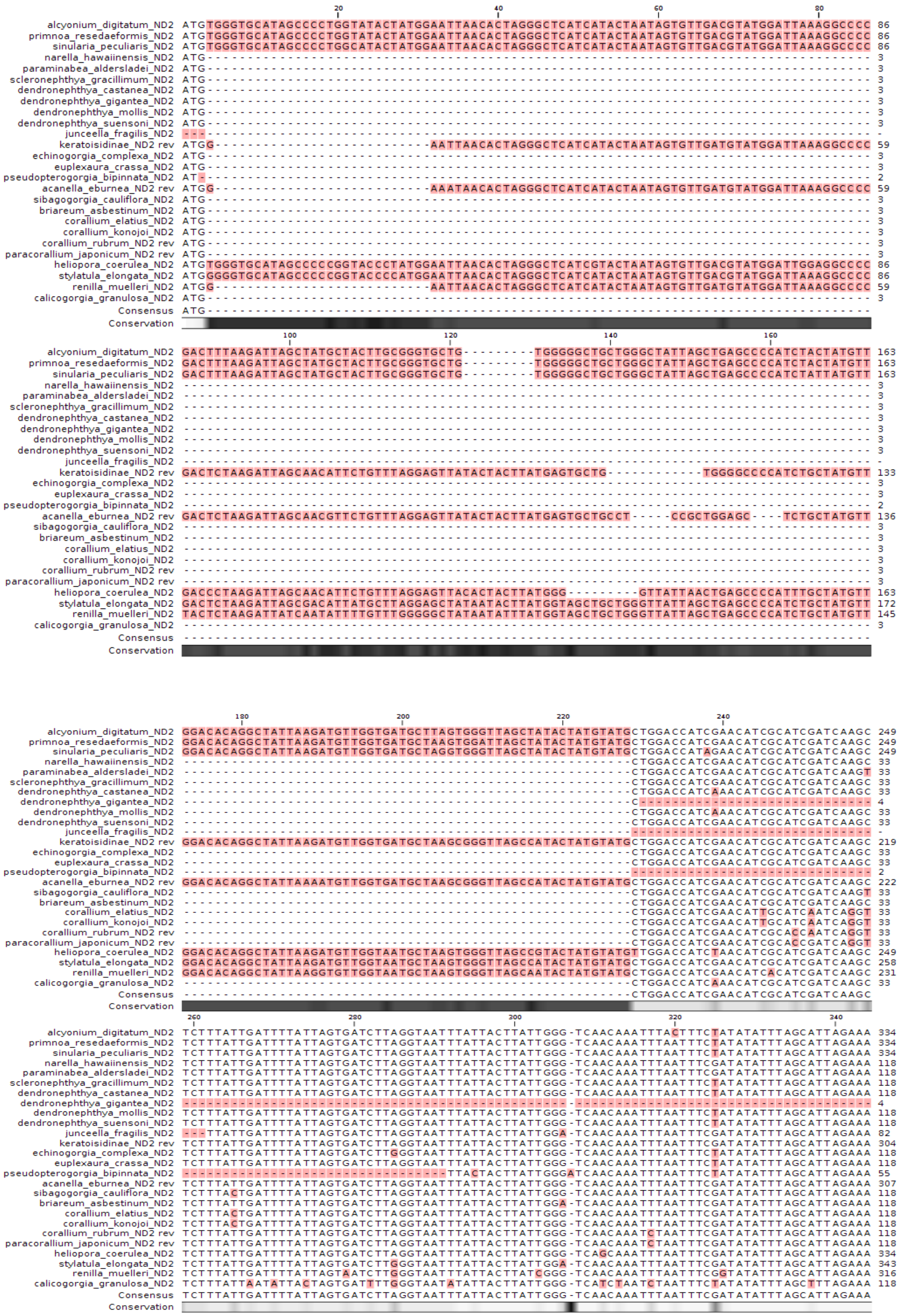

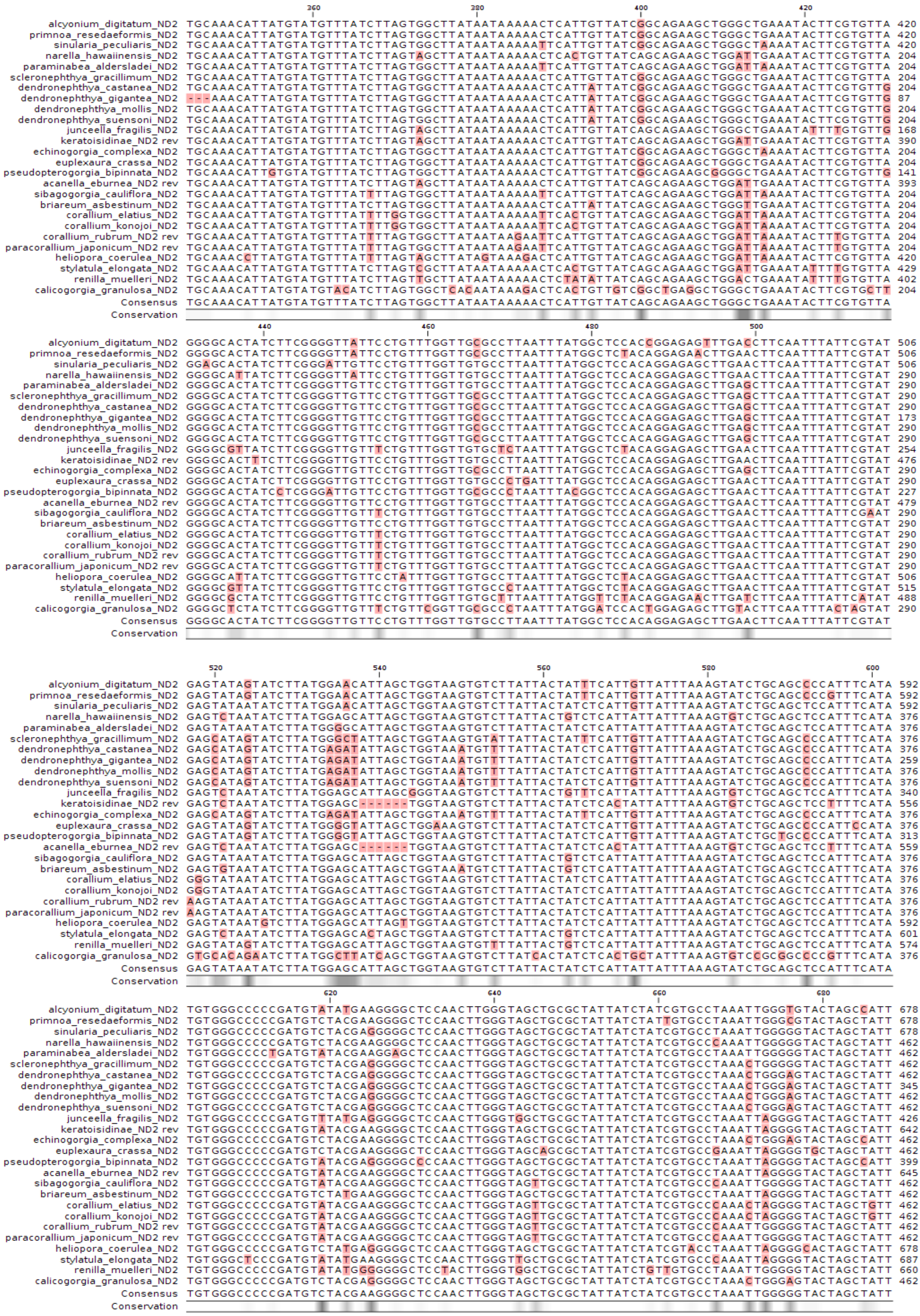

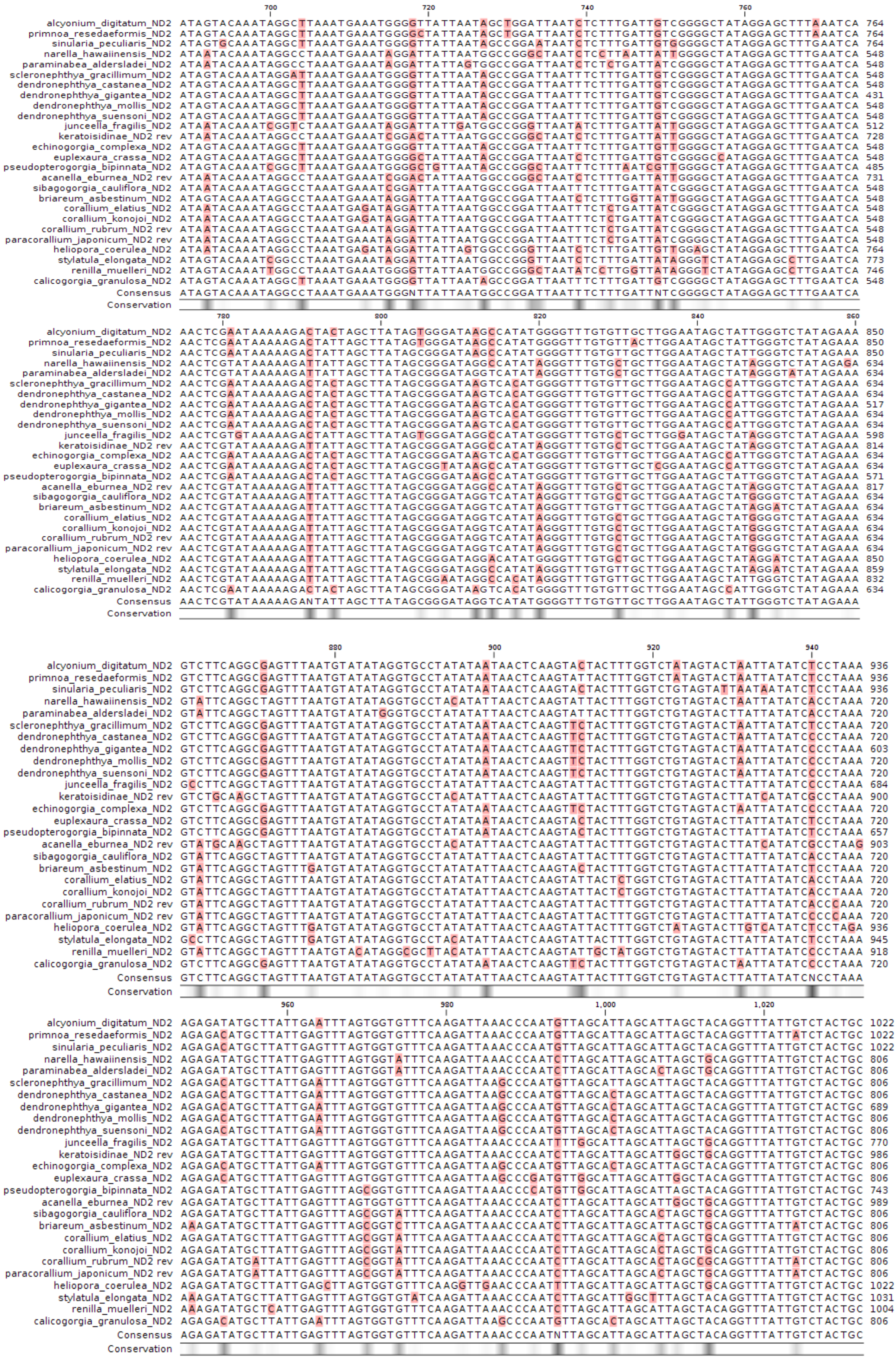

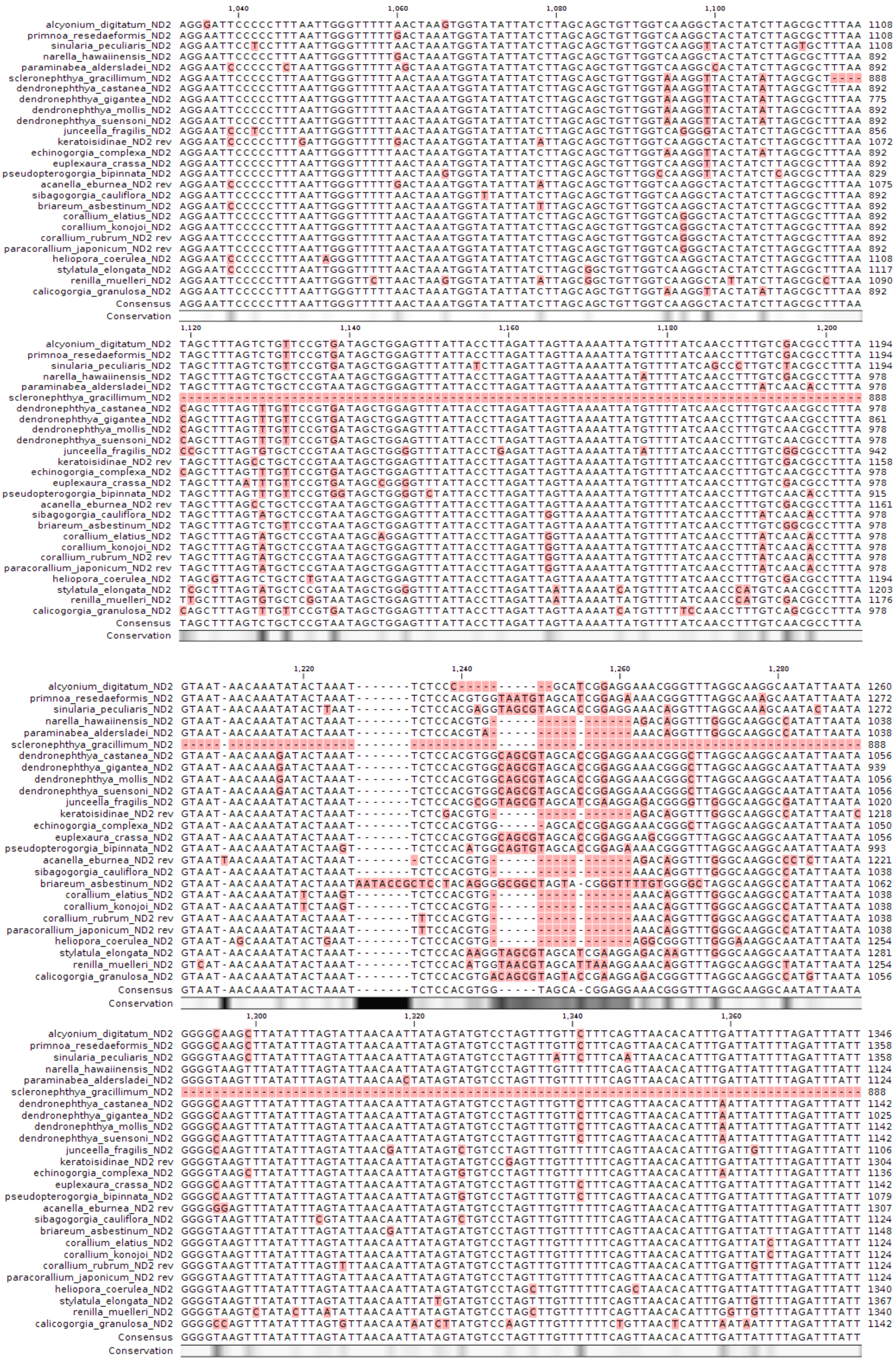

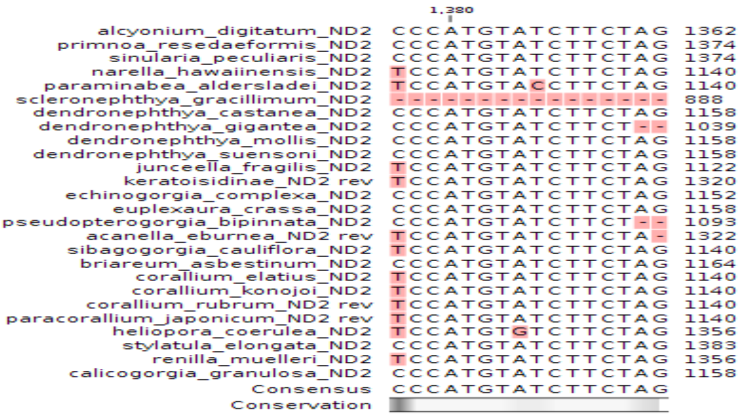

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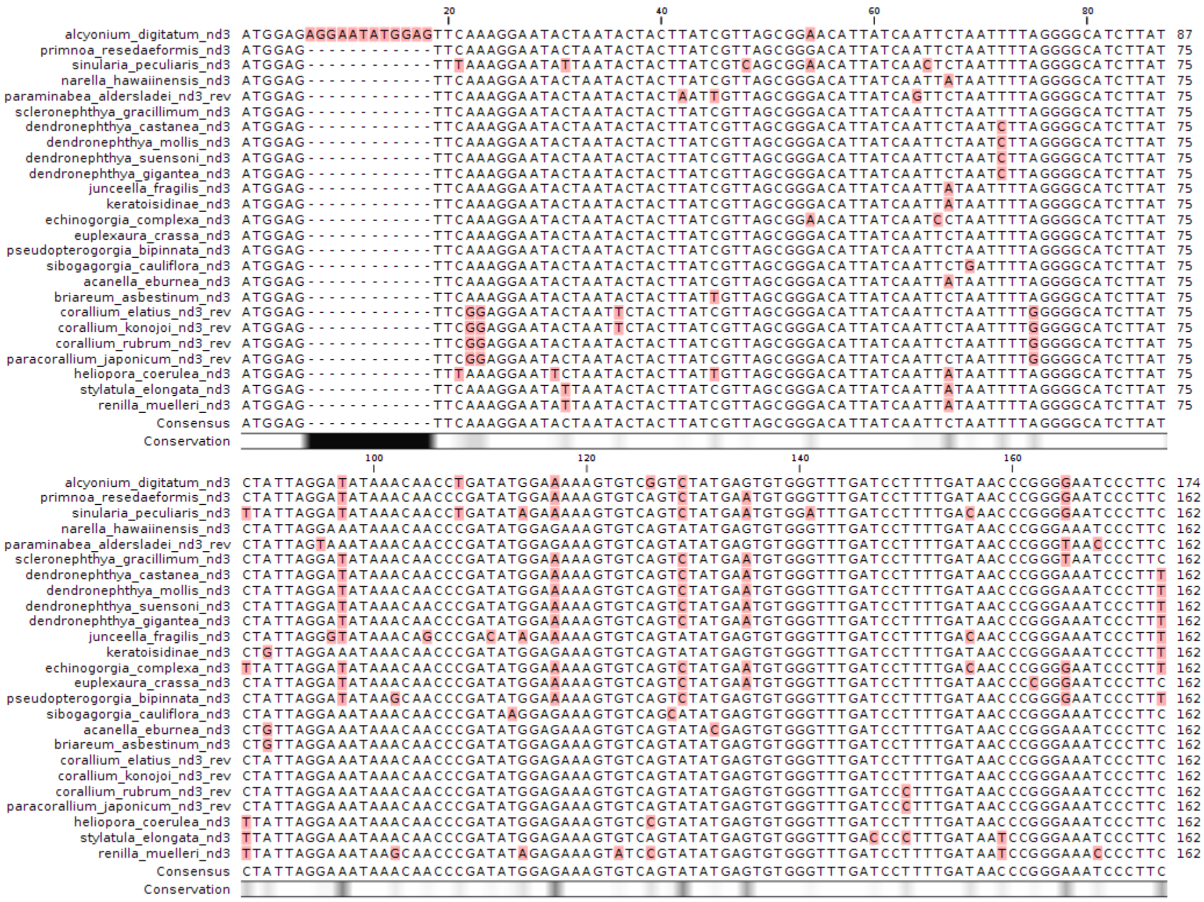

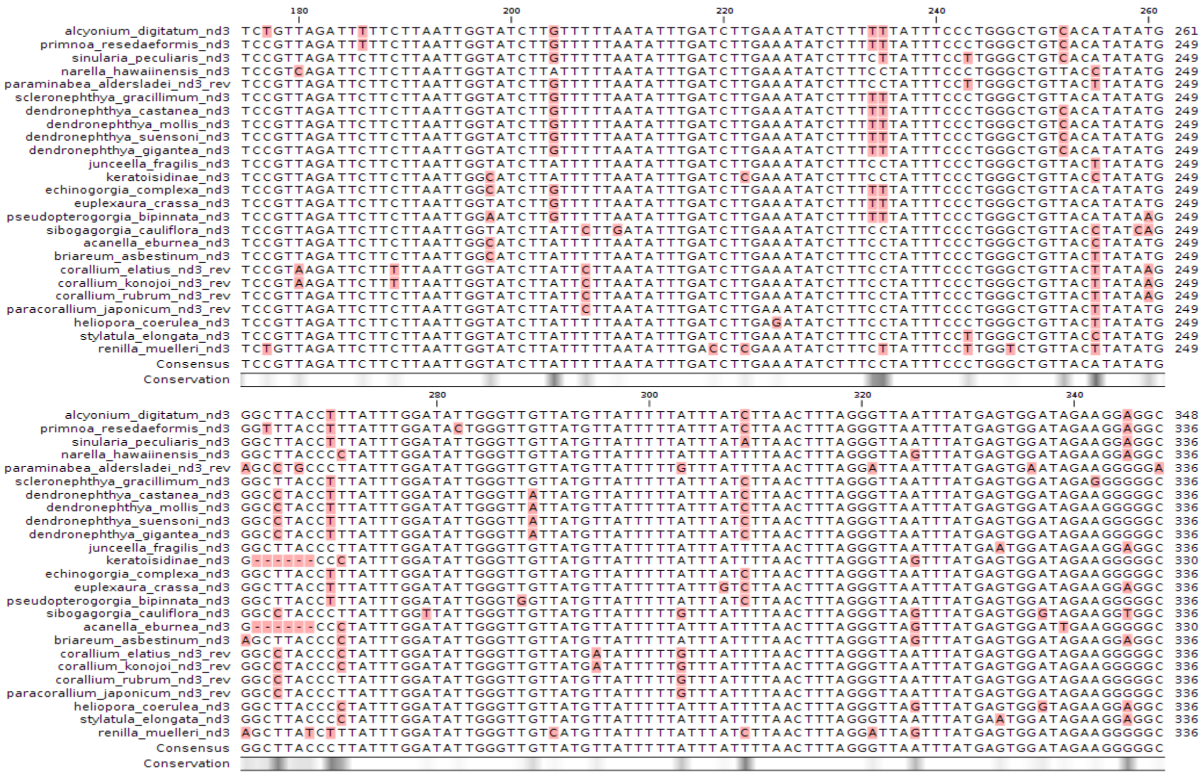

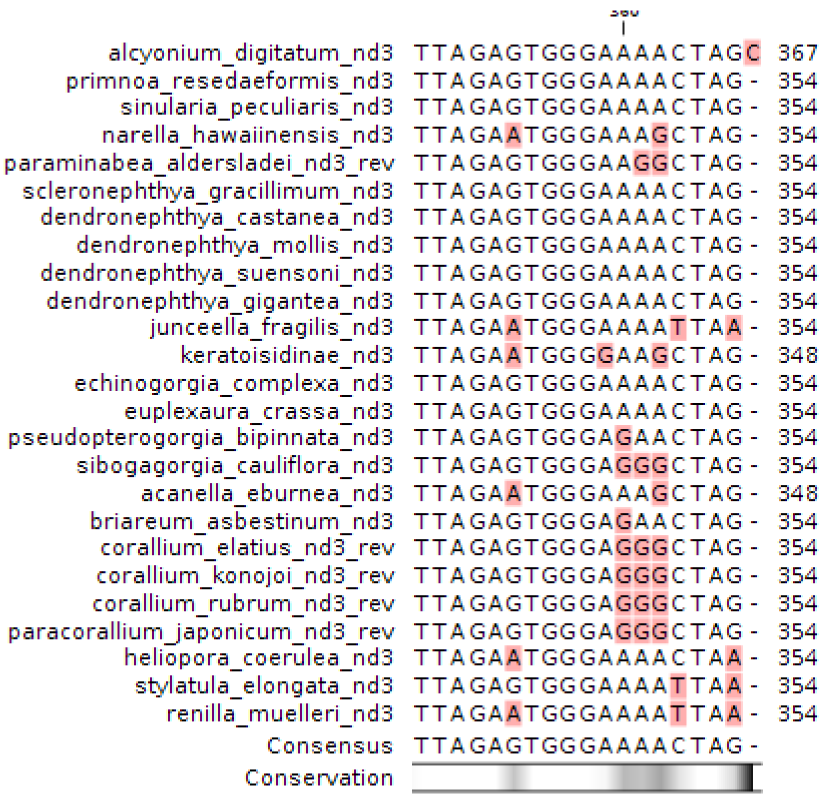
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keratoisidinae_nd3 TTAGAATGGGGAAGCTAG- 348
echinogorgia_complexa_nd3 TTAGAGTGGGAAAACTAG- 354
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corallium_rubrum_nd3_rev TTAGAGTGGGAGGGCTAG- 354
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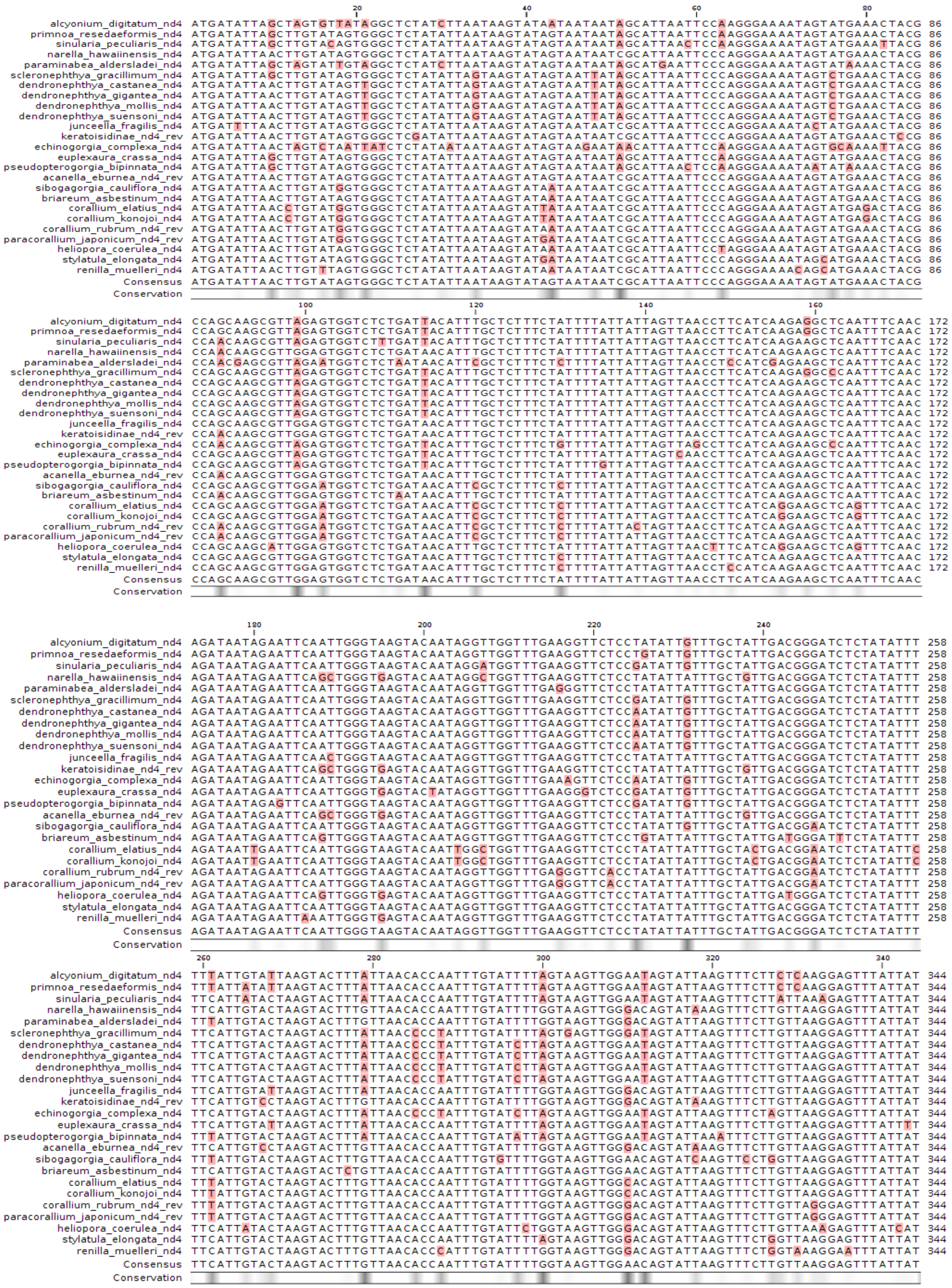

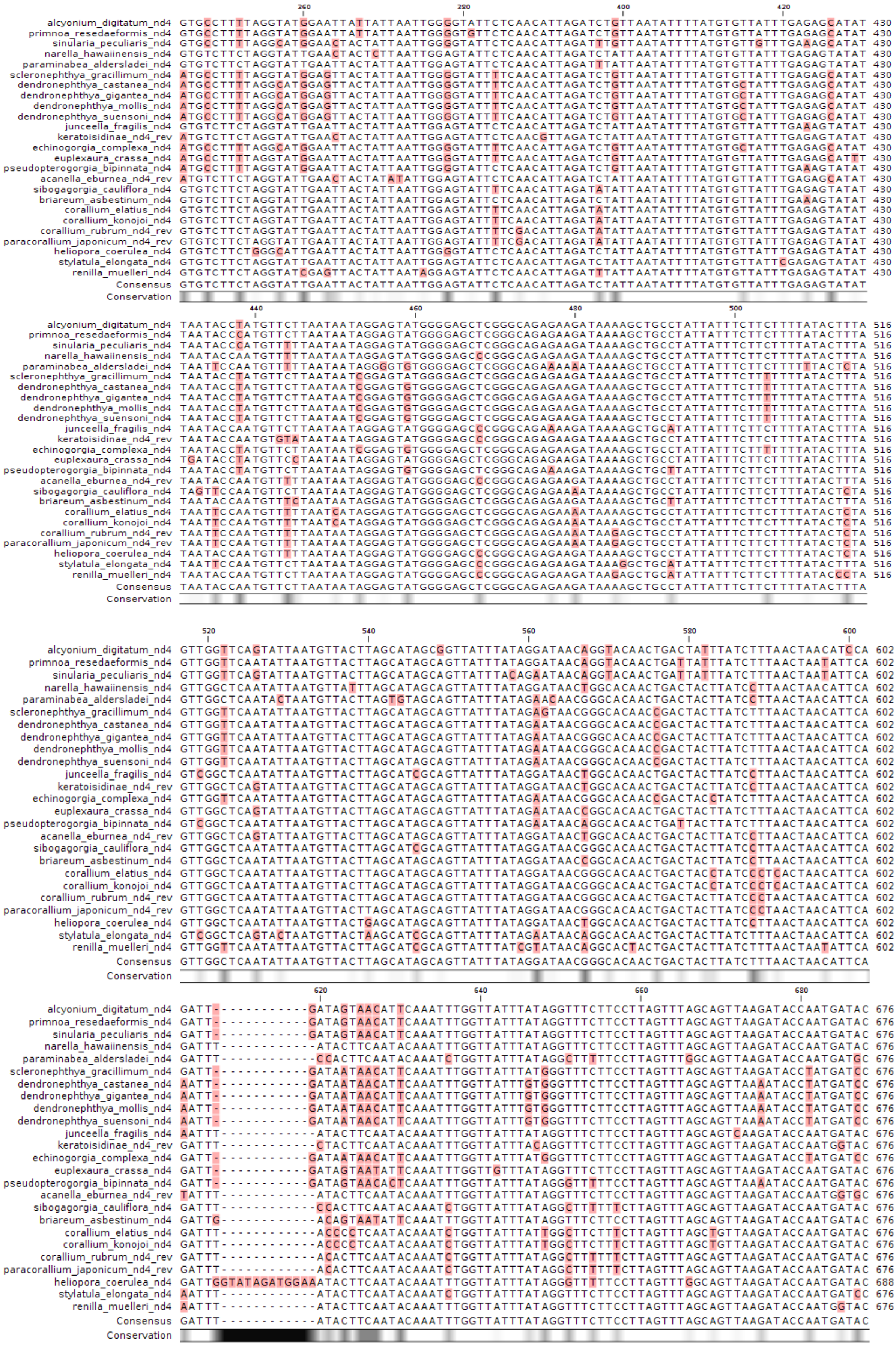

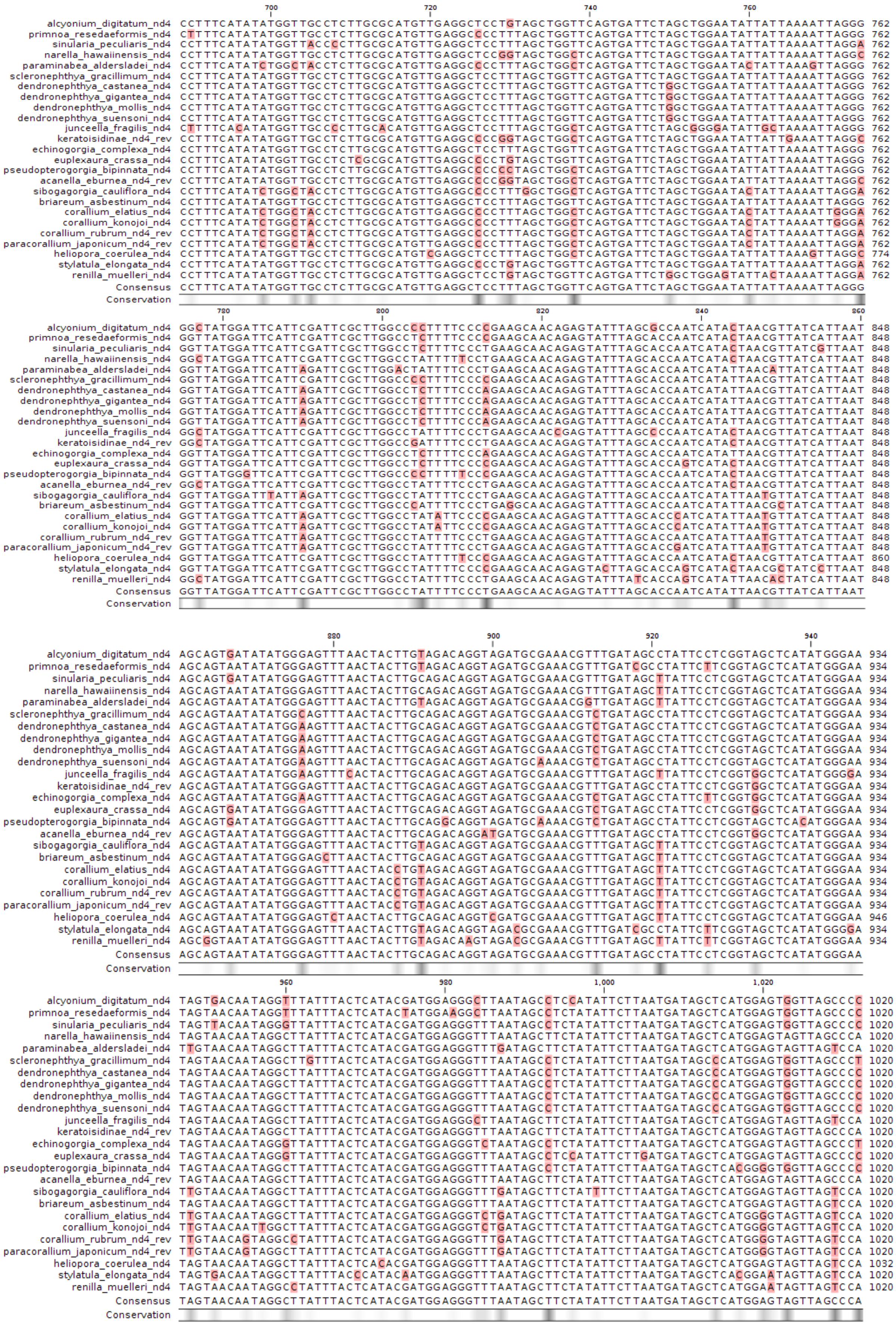

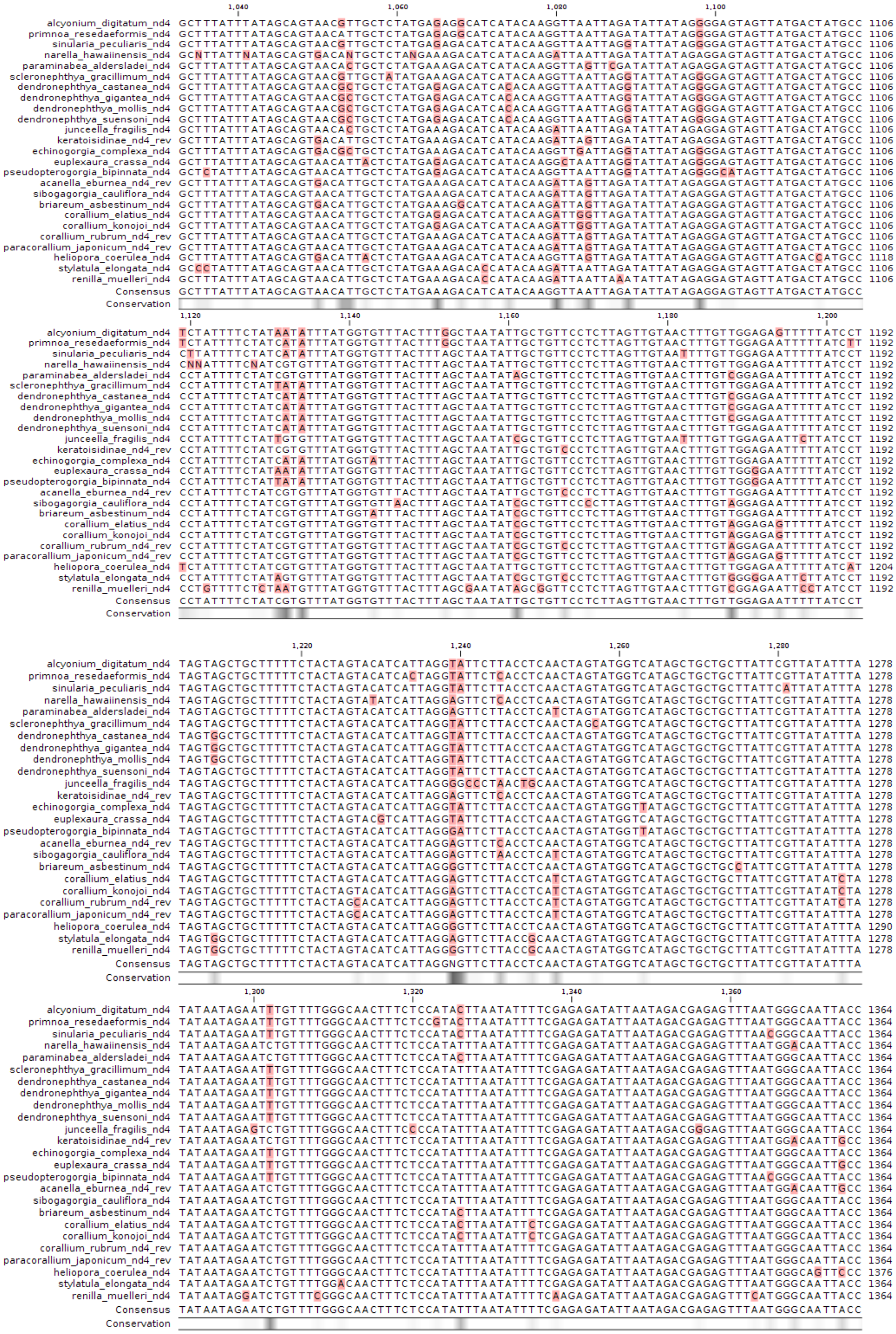

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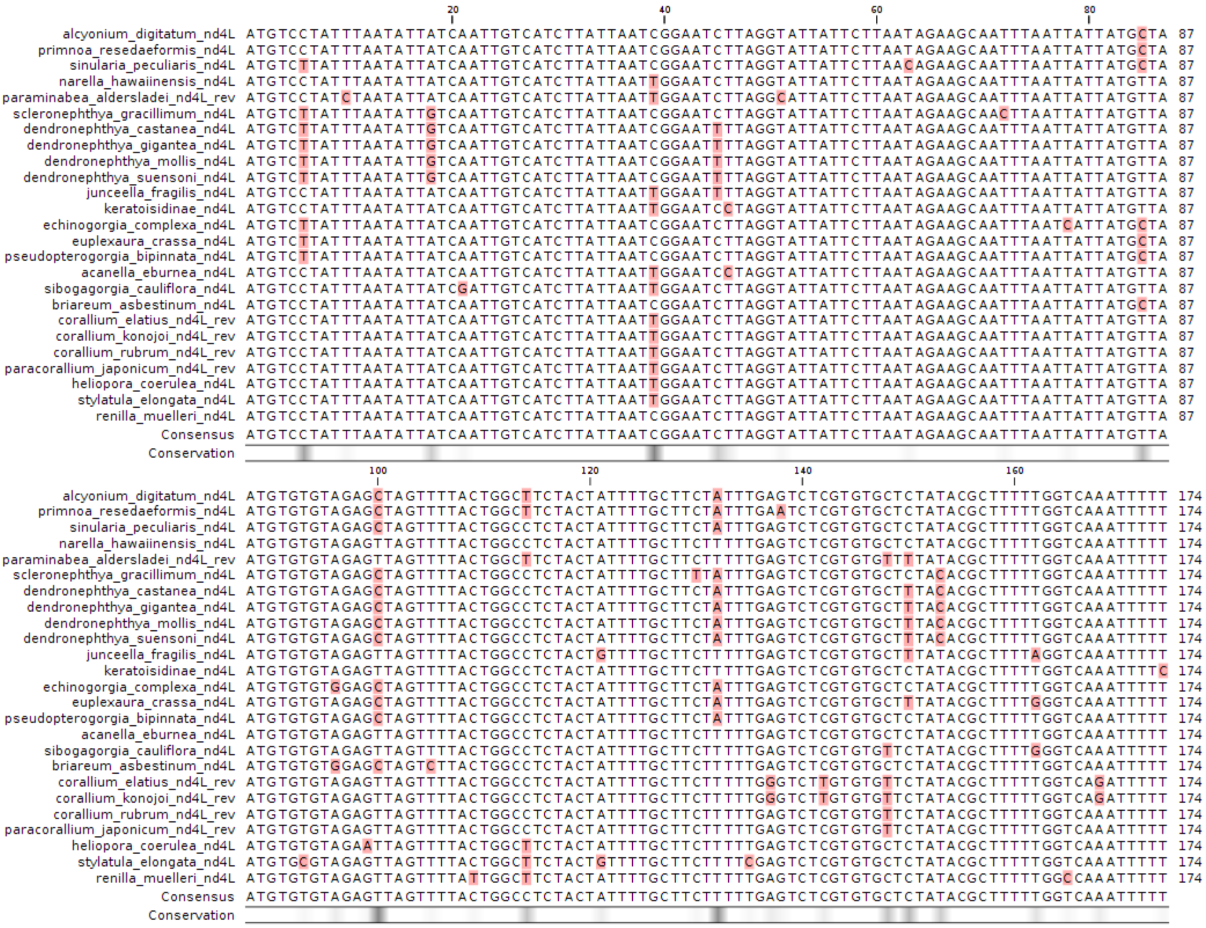

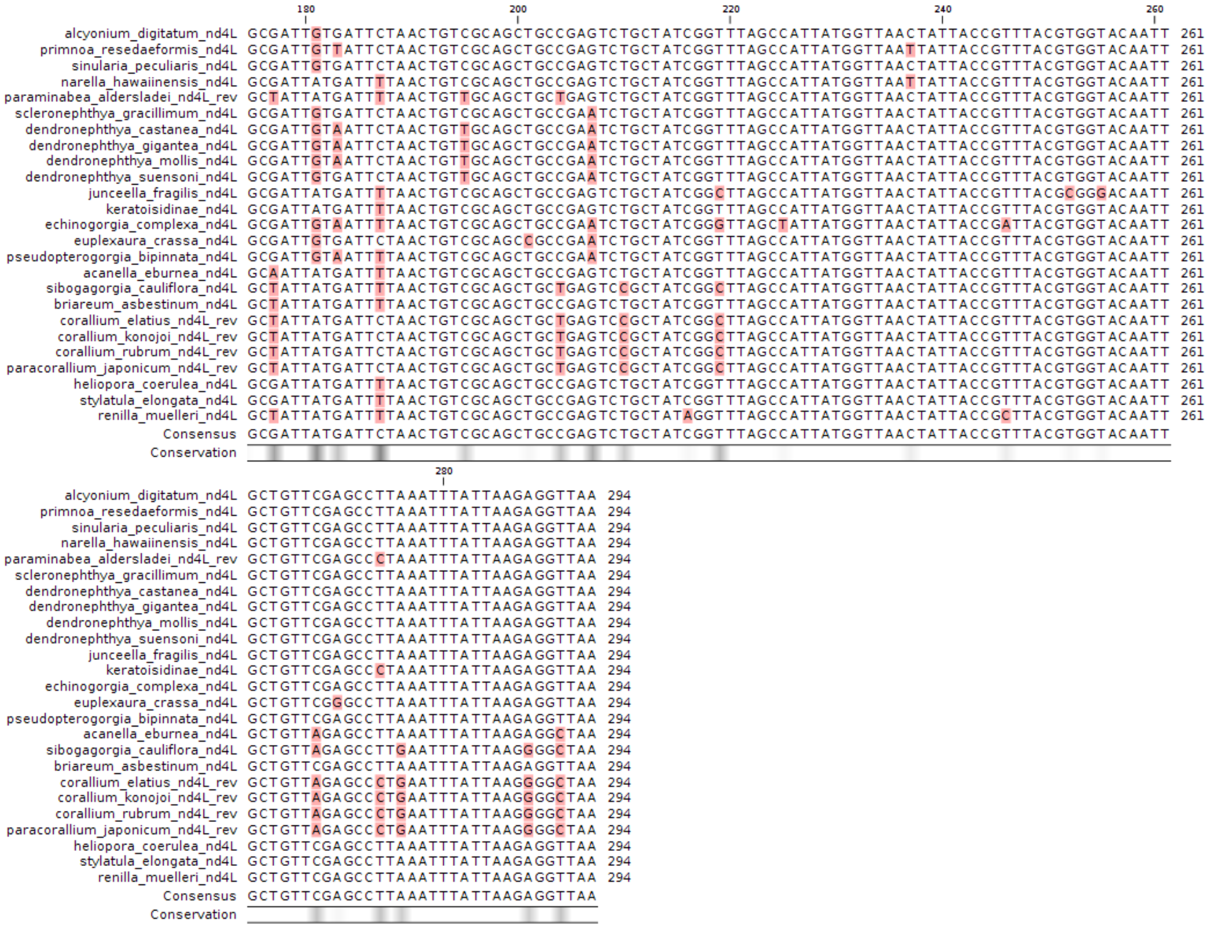

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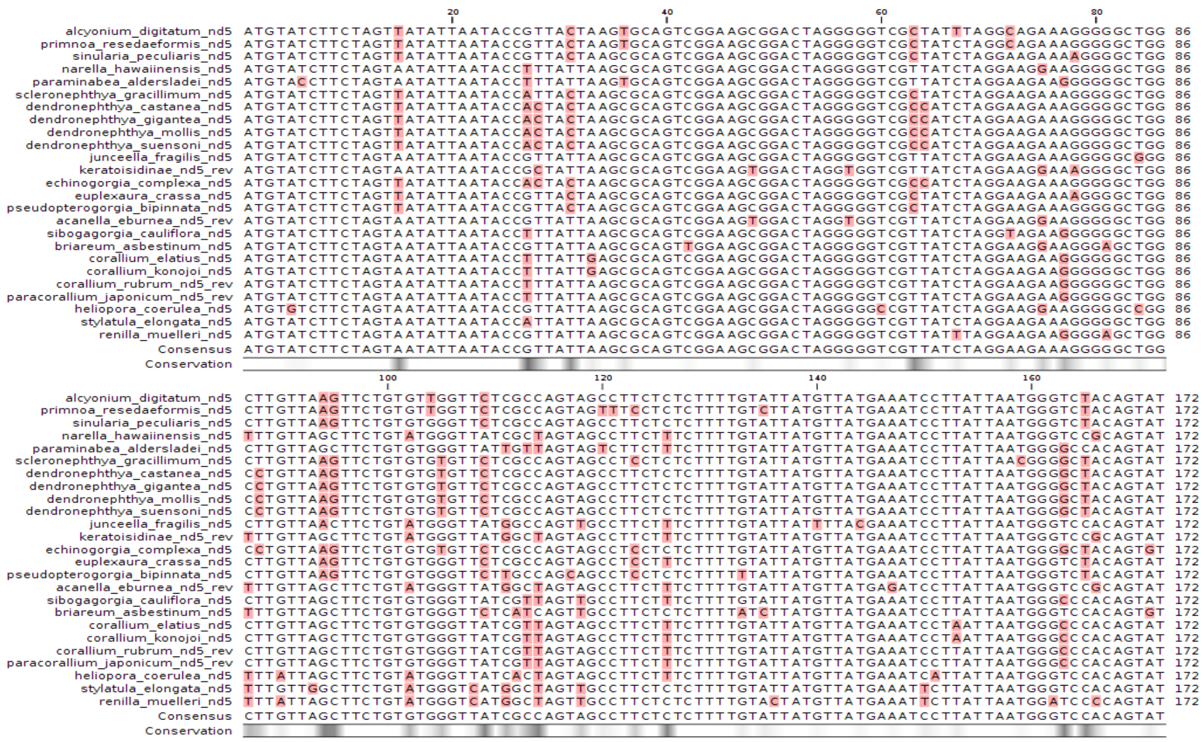

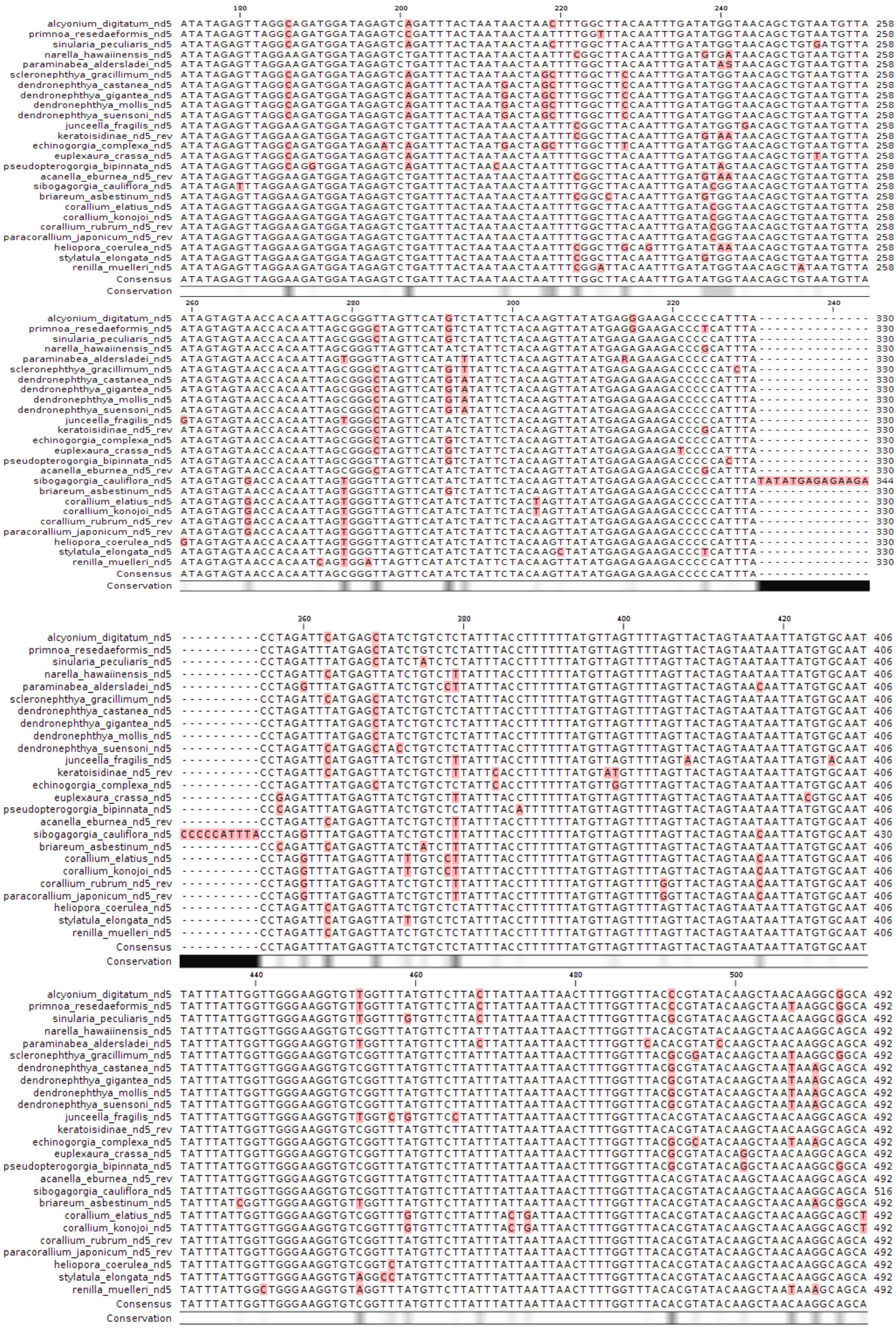

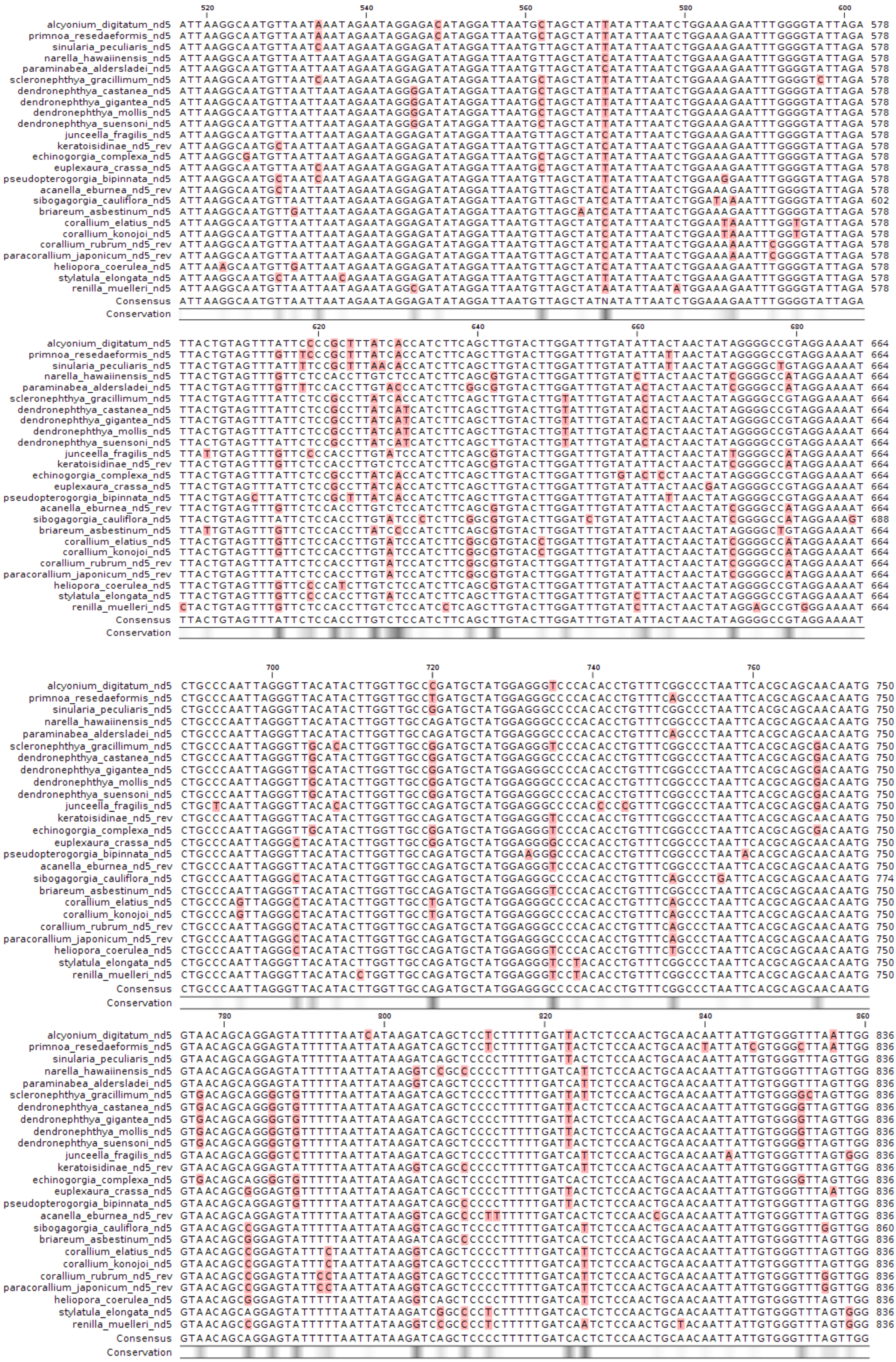
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dendronephthya_castanea_nd5
dendronephthya_gigantea_nd5
dendronephthya_mollis_nd5
dendronephthya_suensoni_nd5
junceella_fragilis_nd5
keratoisidinae_nd5_rev
echinogorgia_complexa_nd5
euplexaura_crassa_nd5
pseudopterogorgia_bipinnata_nd5
acanella_eburnea_nd5_rev
sibogagorgia_cauliflora_nd5
briareum_asbestinum_nd5
corallium_elatius_nd5
corallium_konojoi_nd5
corallium_rubrum_nd5_rev
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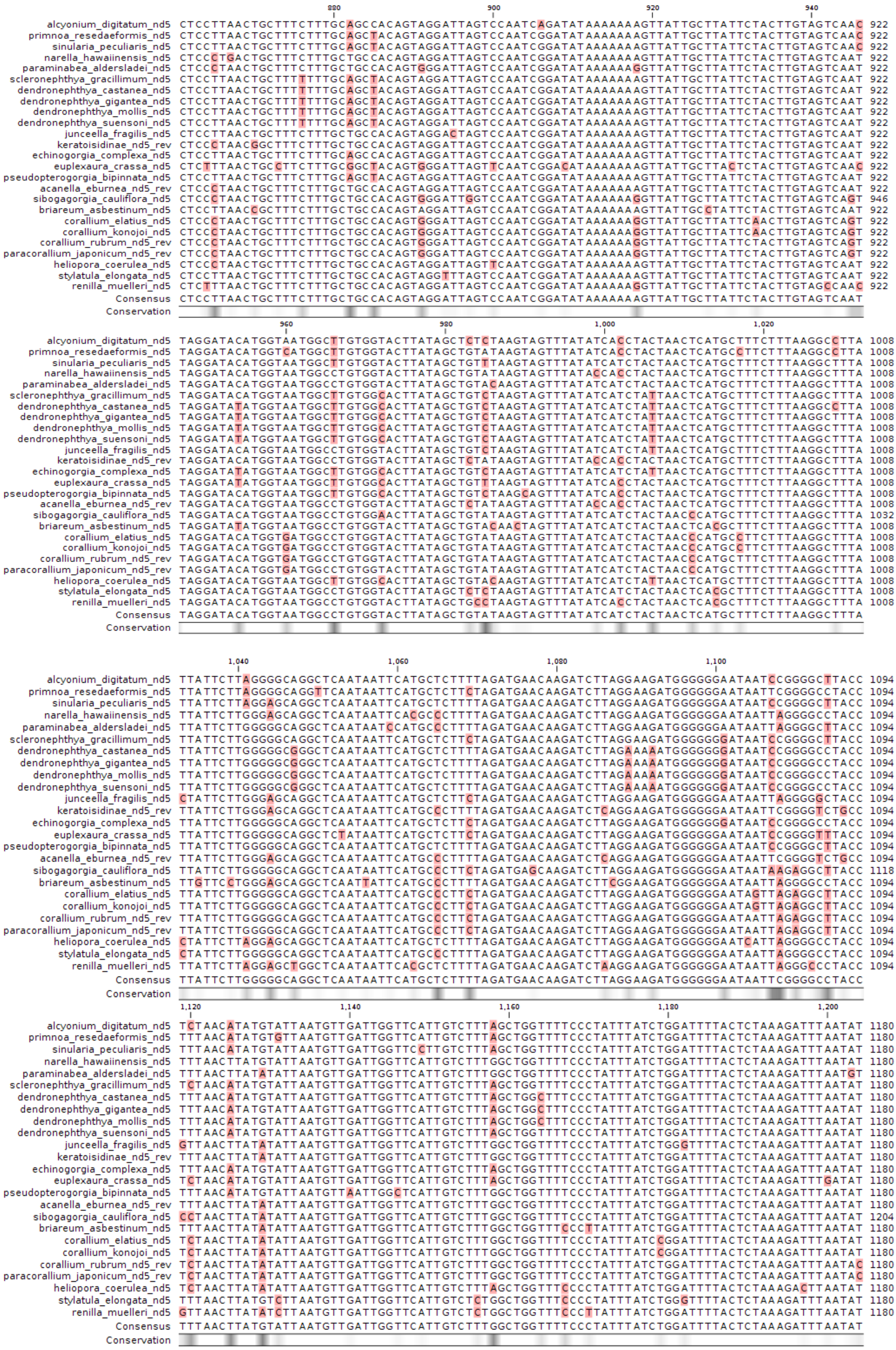
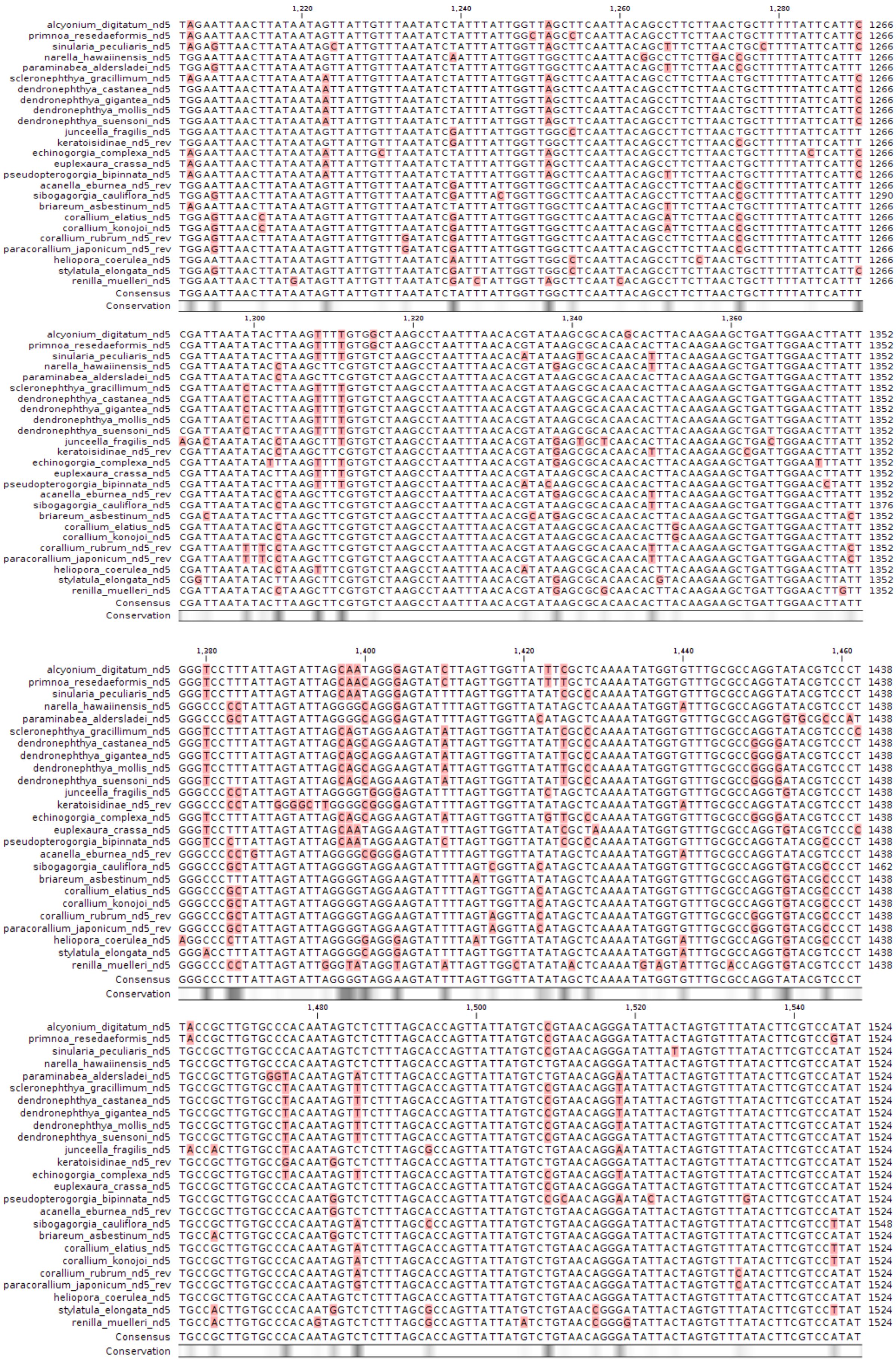
Consensus
Conservation

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dendronephthya_suensoni_nd5
junceella_fragilis_nd5
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echinogorgia_complexa_nd5
euplexaura_crassa_nd5
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acanella_eburnea_nd5_rev
sibogagorgia_cauliflora_nd5
briareum_asbestinum_nd5
corallium_elatius_nd5
corallium_konojoi_nd5
corallium_rubrum_nd5_rev
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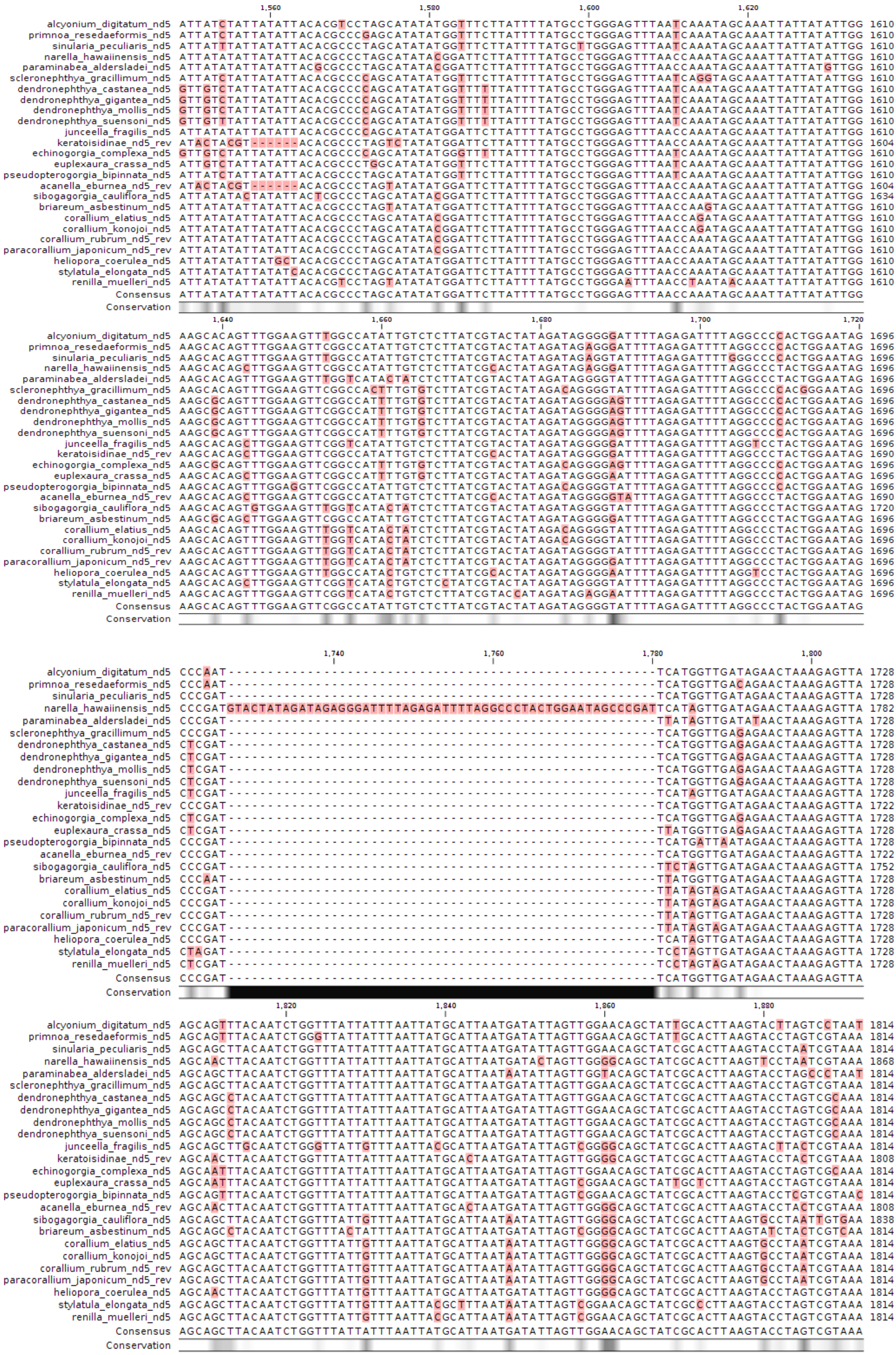
Conservation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dendronephthya_gigantea_nd5
dendronephthya_mollis_nd5
dendronephthya_suensoni_nd5
junceella_fragilis_nd5
keratoisidinae_nd5_rev
echinogorgia_complexa_nd5
euplexaura_crassa_nd5
pseudopterogorgia_bipinnata_nd5
acanella_eburnea_nd5_rev
sibogagorgia_cauliflora_nd5
briareum_asbestinum_nd5
corallium_elatius_nd5
corallium_konojoi_nd5
corallium_rubrum_nd5_rev
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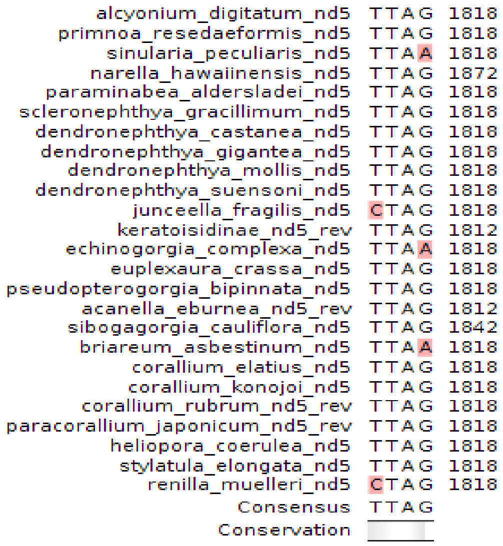
Conservation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Consensus TTAG
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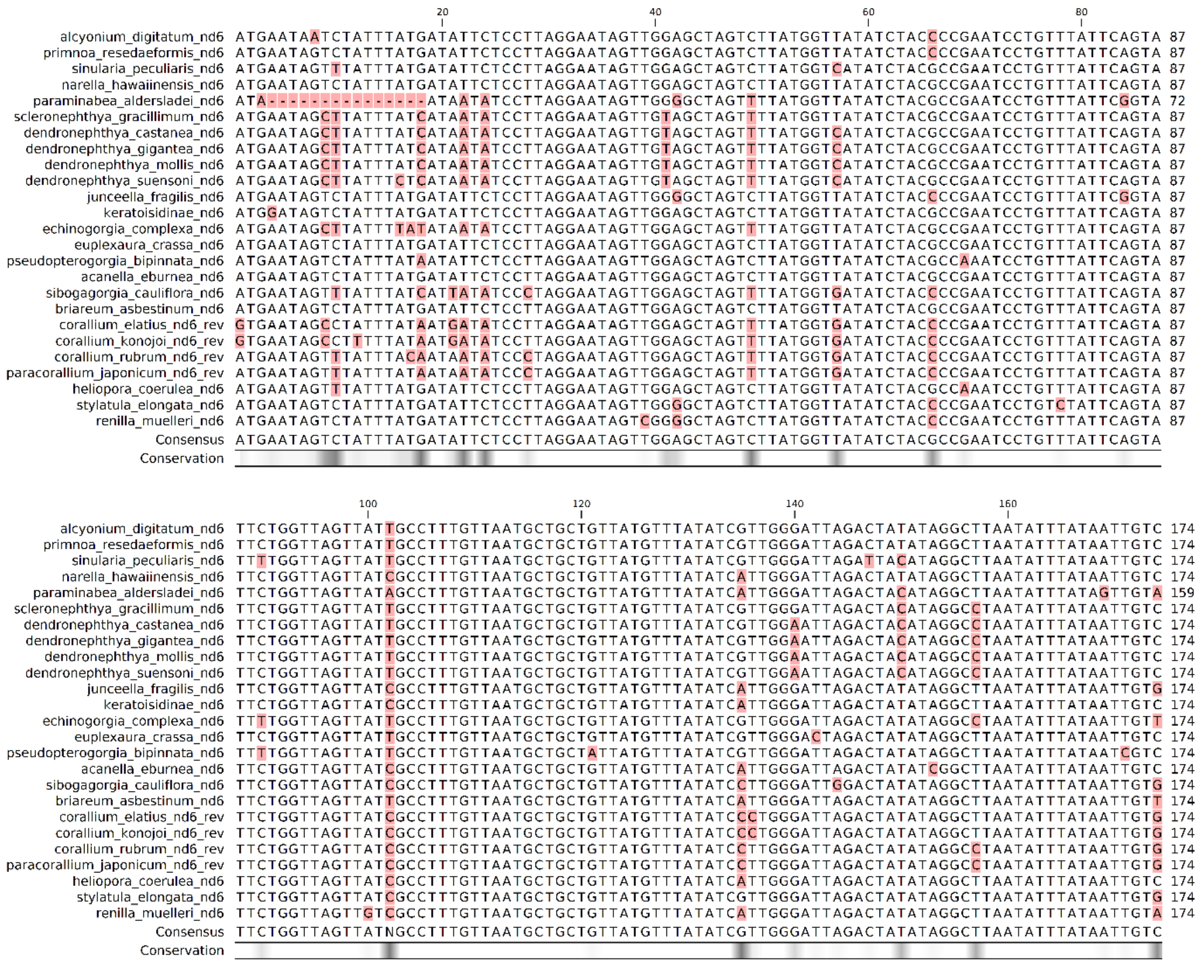
Nd6 gene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scleronephthya_gracillimum_nd6
dendronephthya_castanea_nd6
dendronephthya_gigantea_nd6
dendronephthya_mollis_nd6
dendronephthya_suensoni_nd6
junceella_fragilis_nd6
keratoisidinae_nd6
echinogorgia_complexa_nd6
euplexaura_crassa_nd6
pseudopterogorgia_bipinnata_nd6
acanella_eburnea_nd6
sibogagorgia_cauliflora_nd6
briareum_asbestinum_nd6
corallium_elatius_nd6_rev
corallium_konojoi_nd6_rev
corallium_rubrum_nd6_rev
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heliopora_coerulea_nd6
stylatula_elongata_nd6
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Conservation
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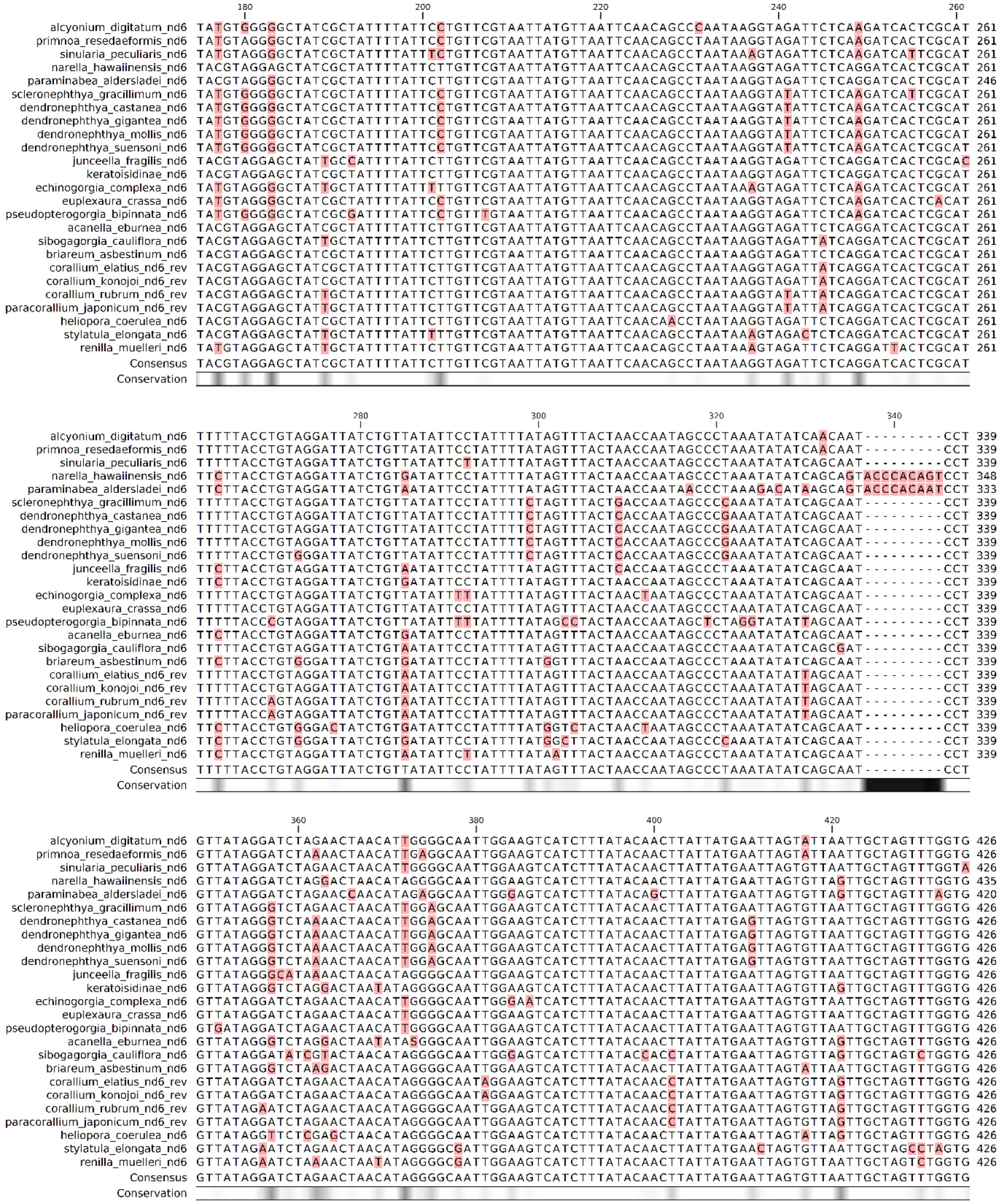
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junceella_fragilis_nd6
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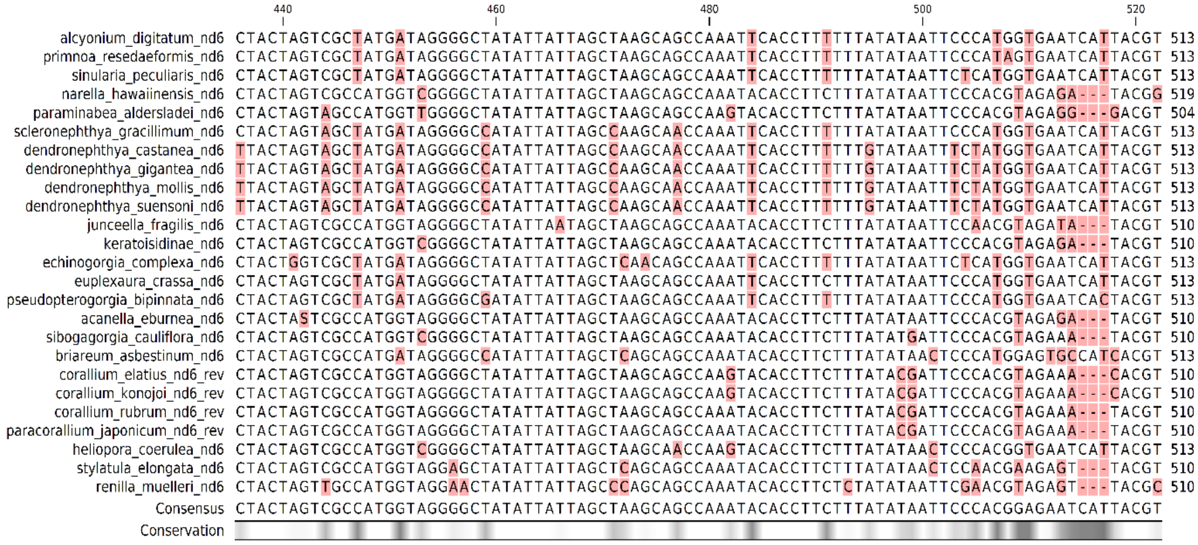
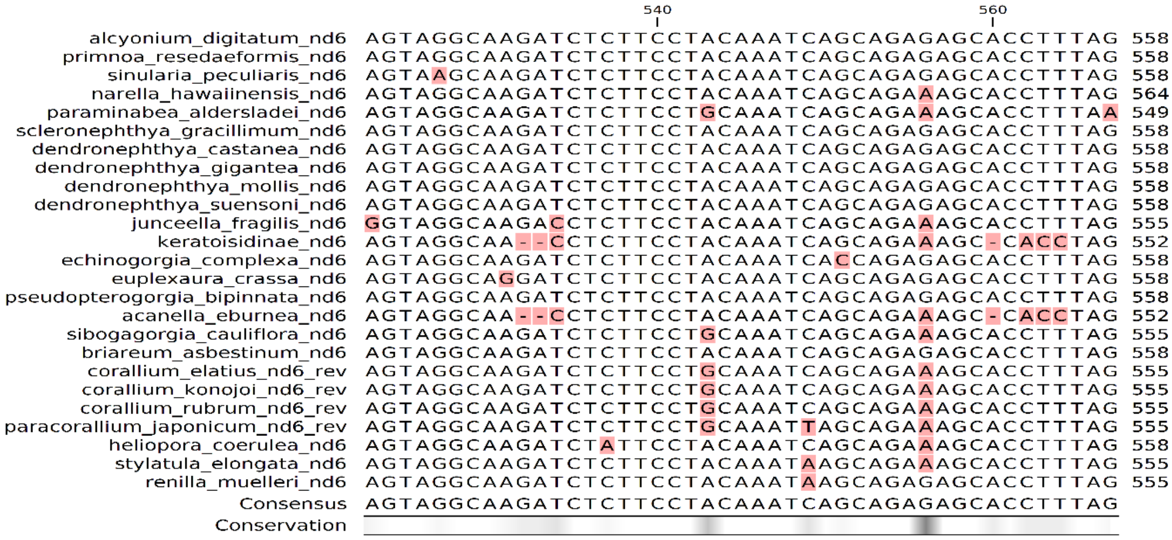
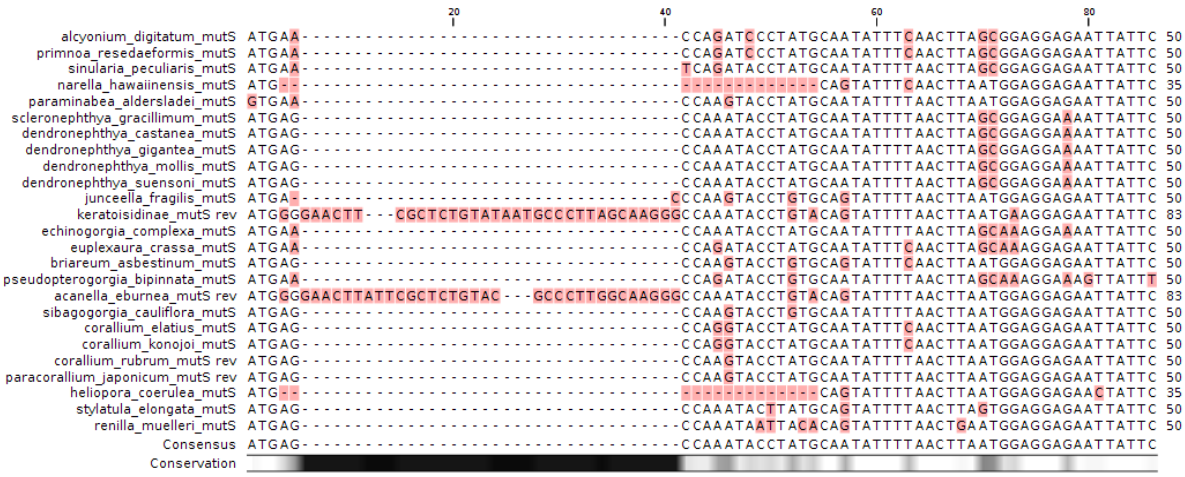
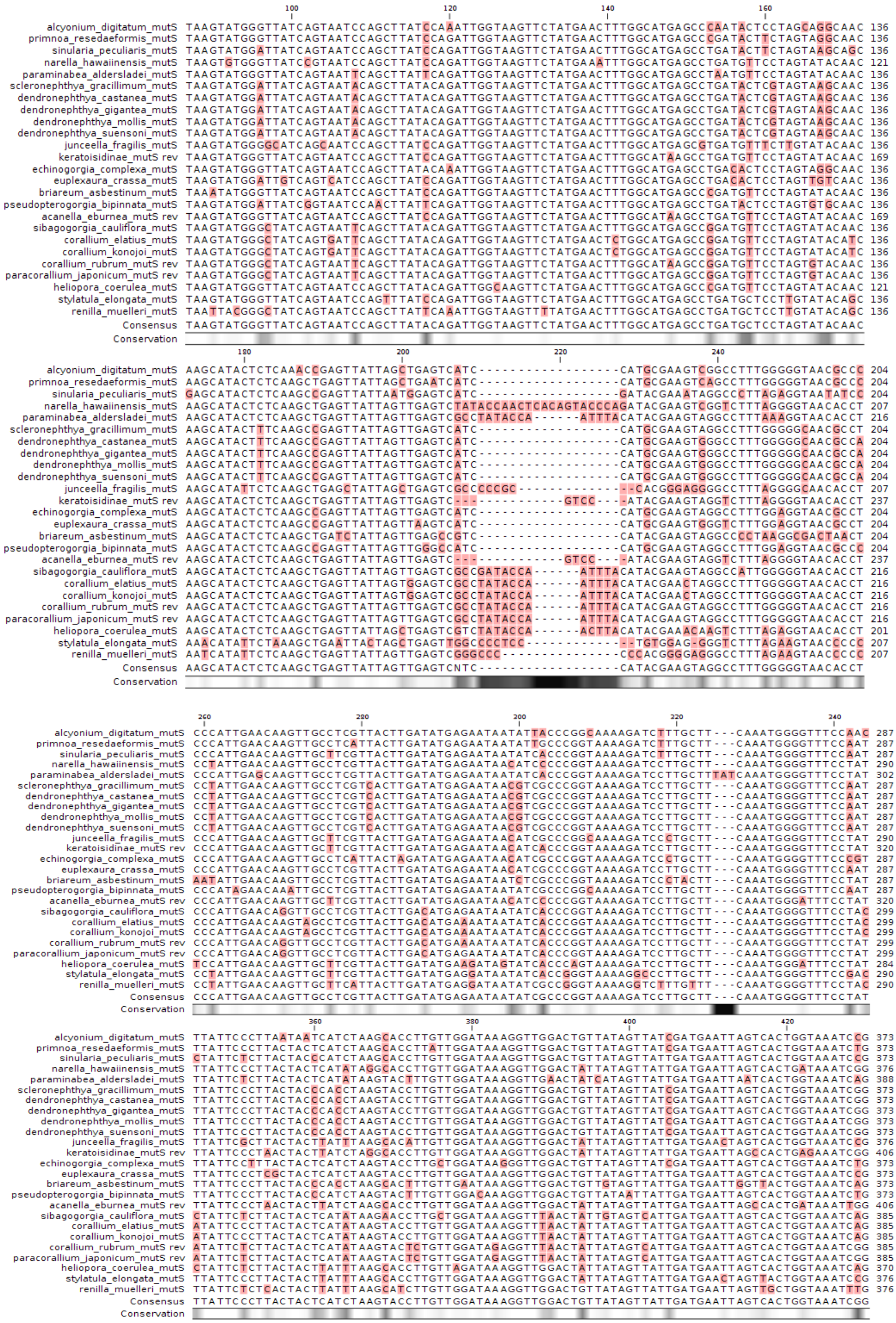
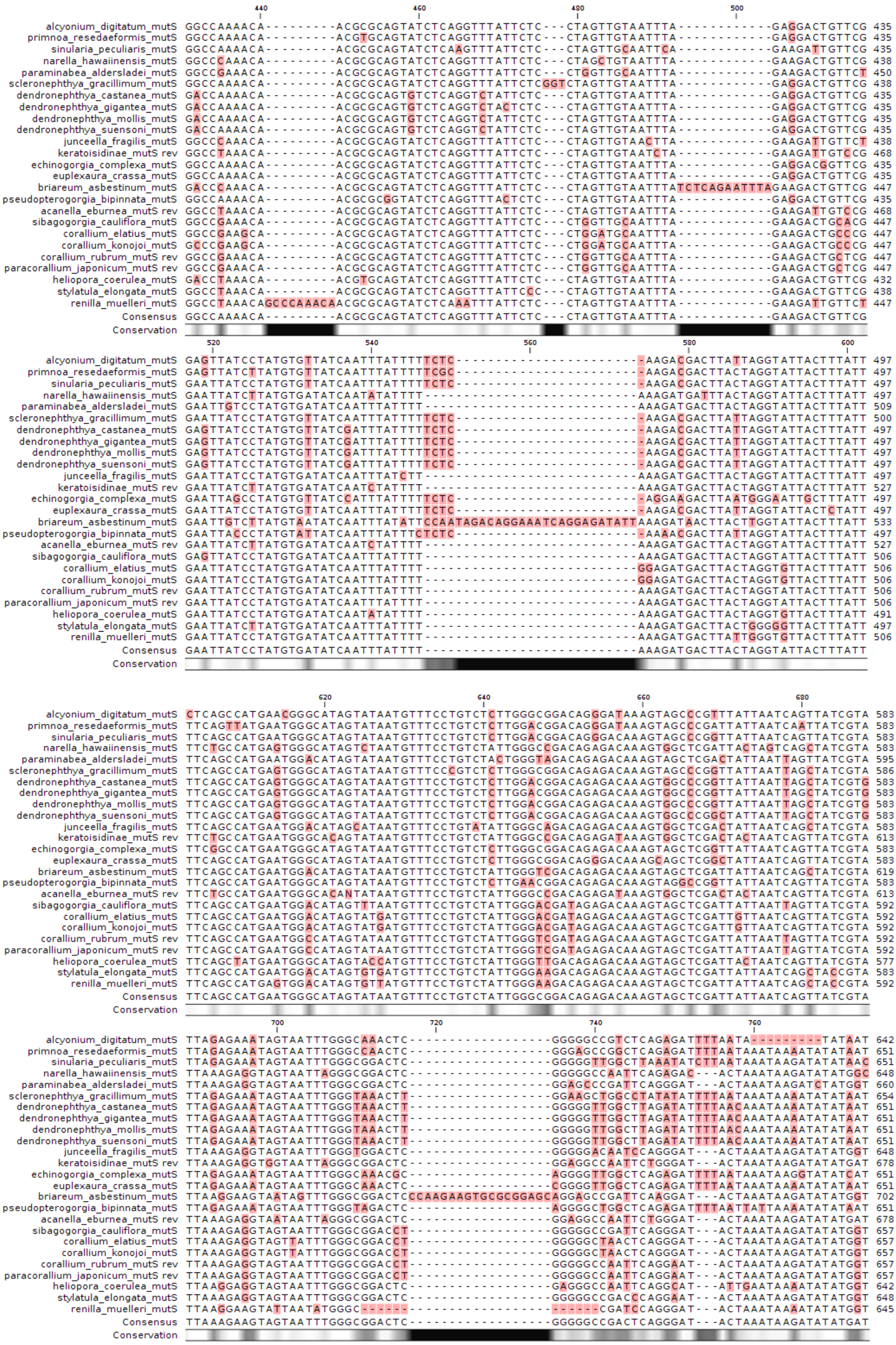


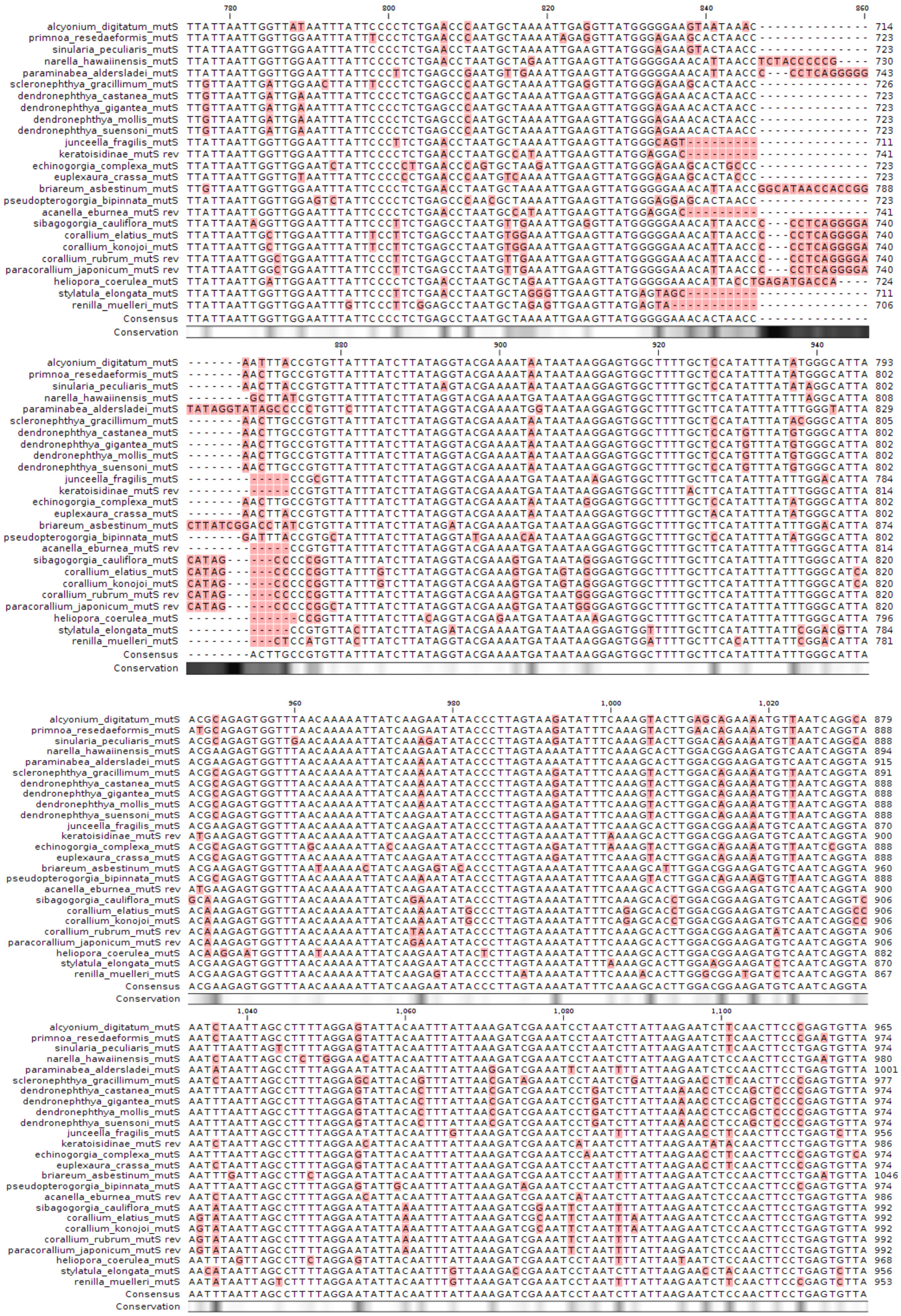


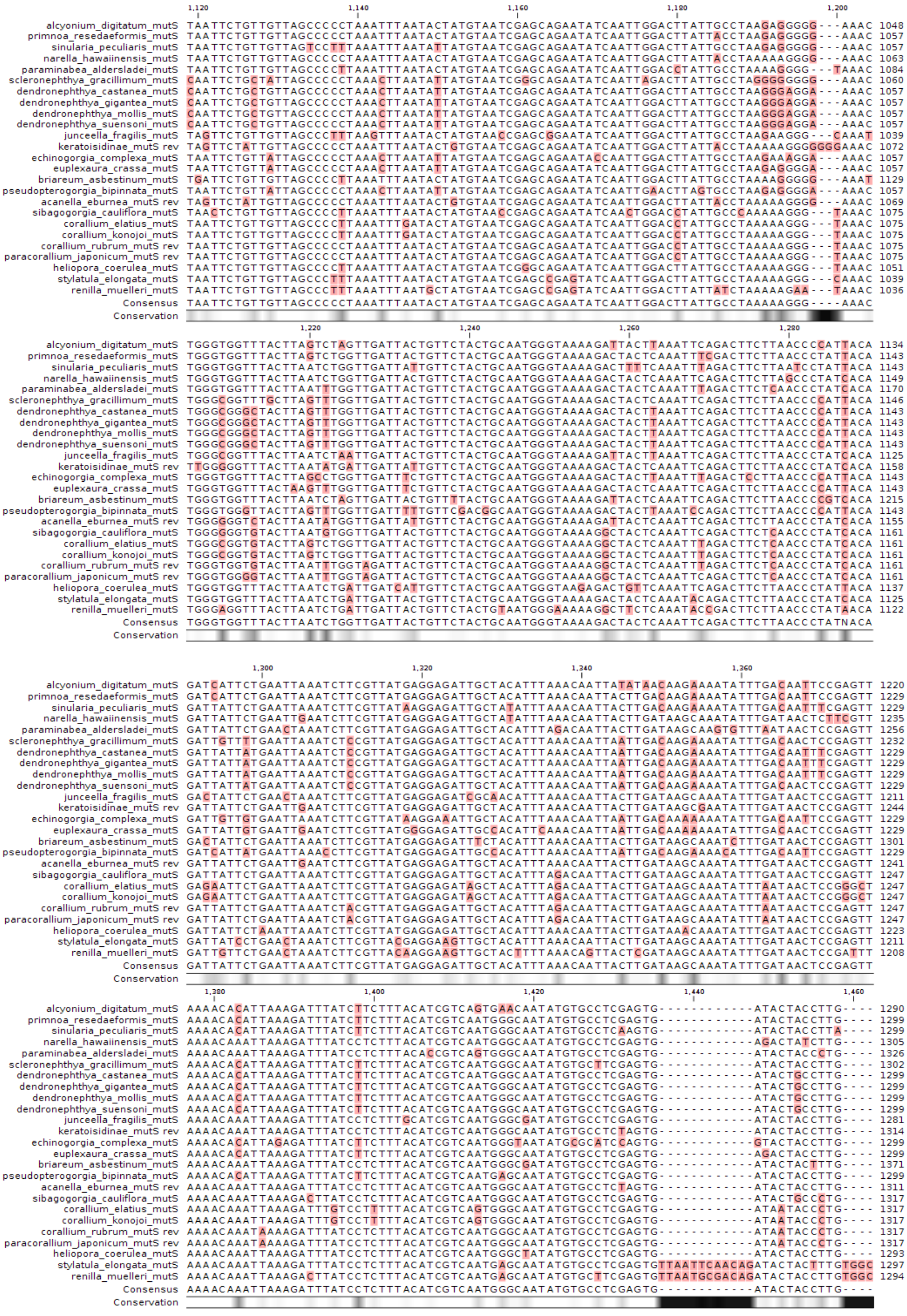
MutS gene







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sibagogorgia_cauliflora_mutS
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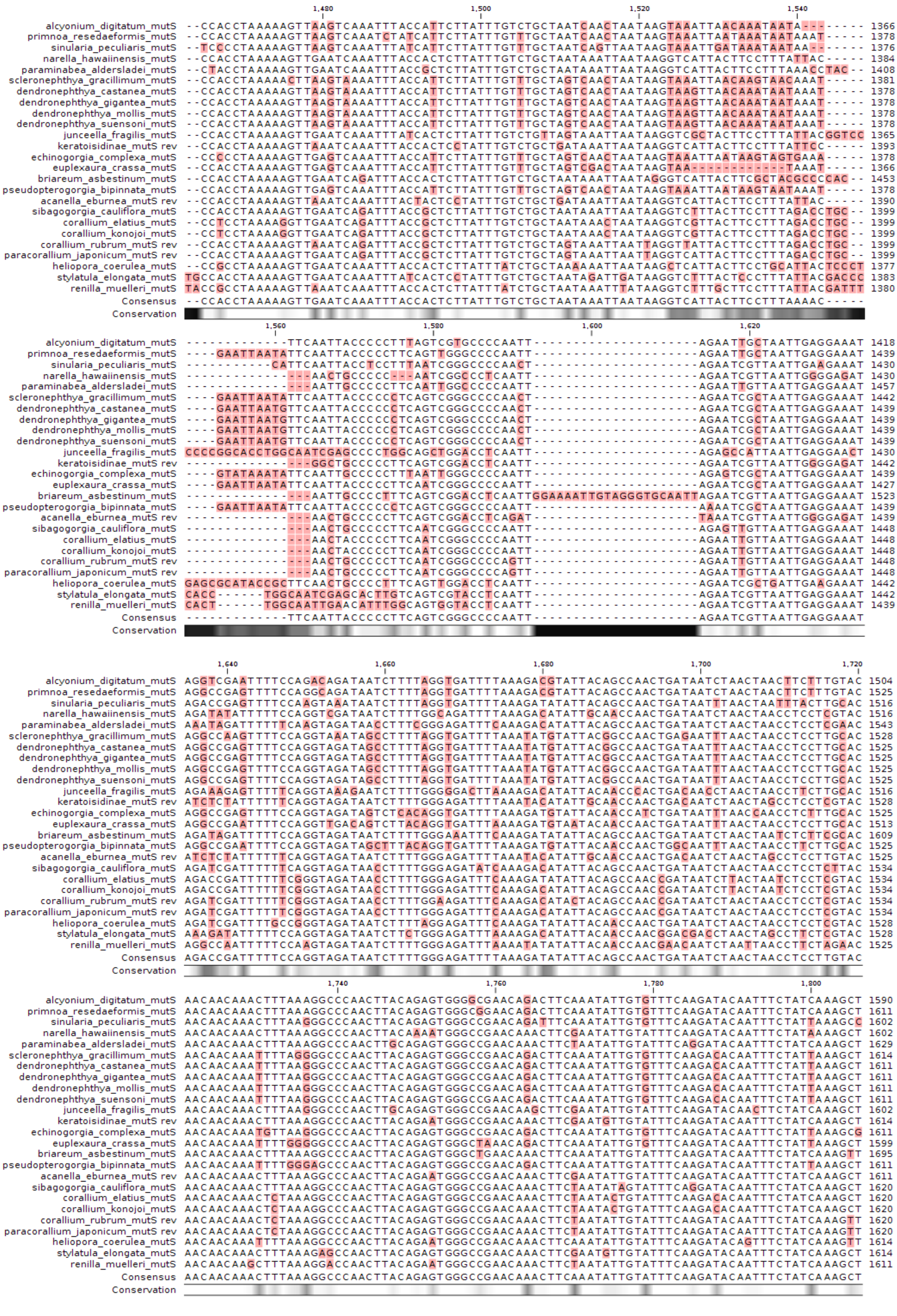
Conservation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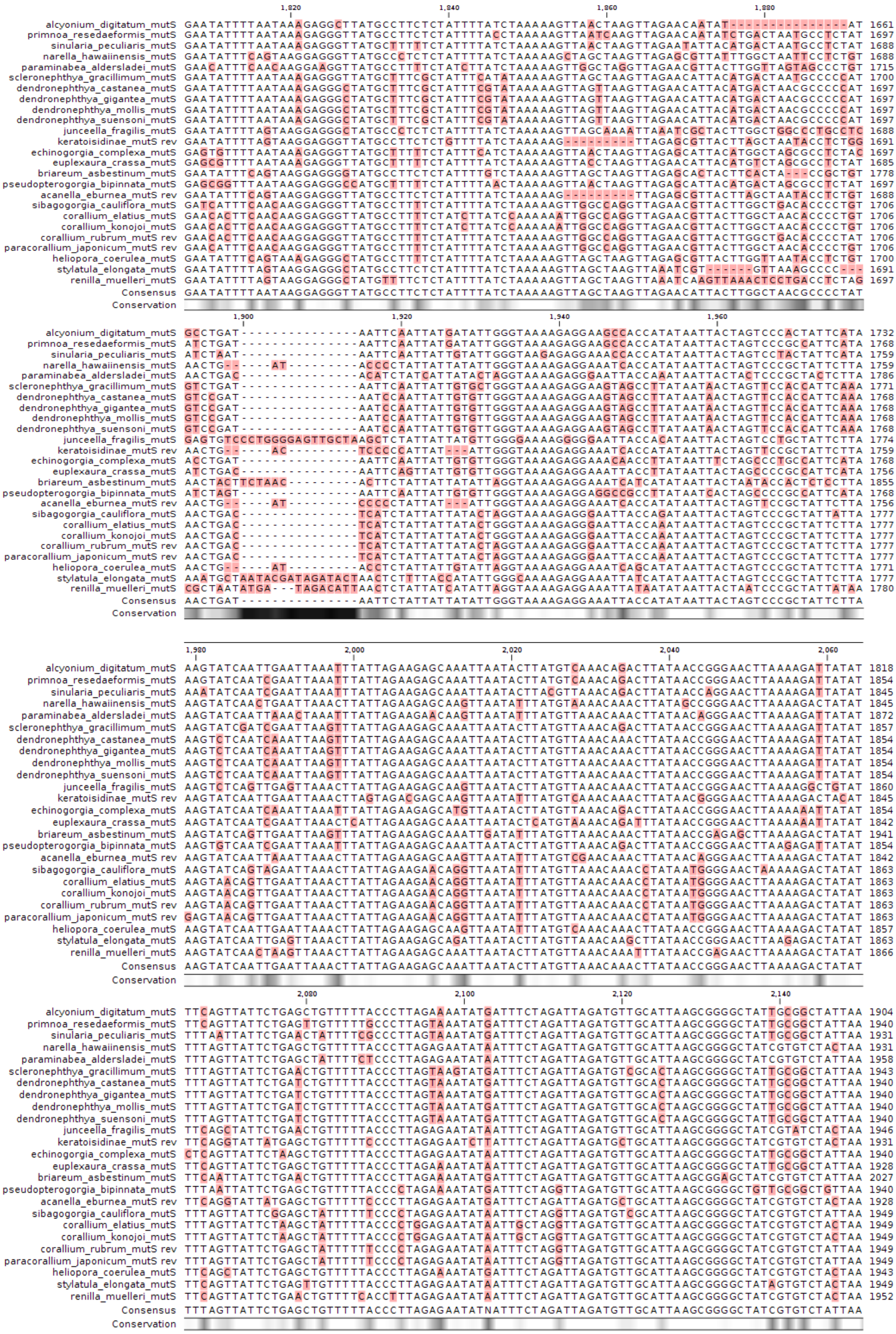
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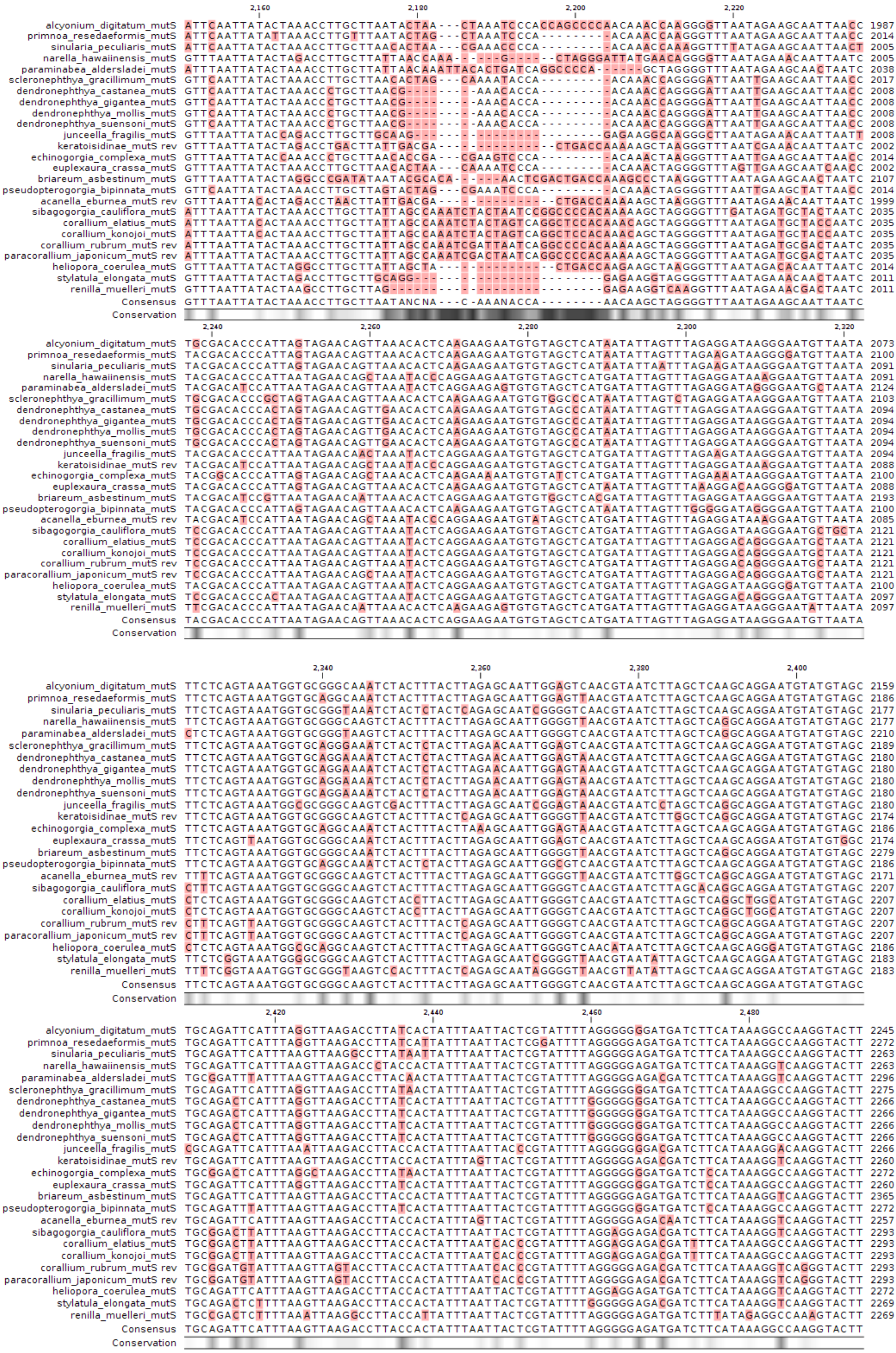
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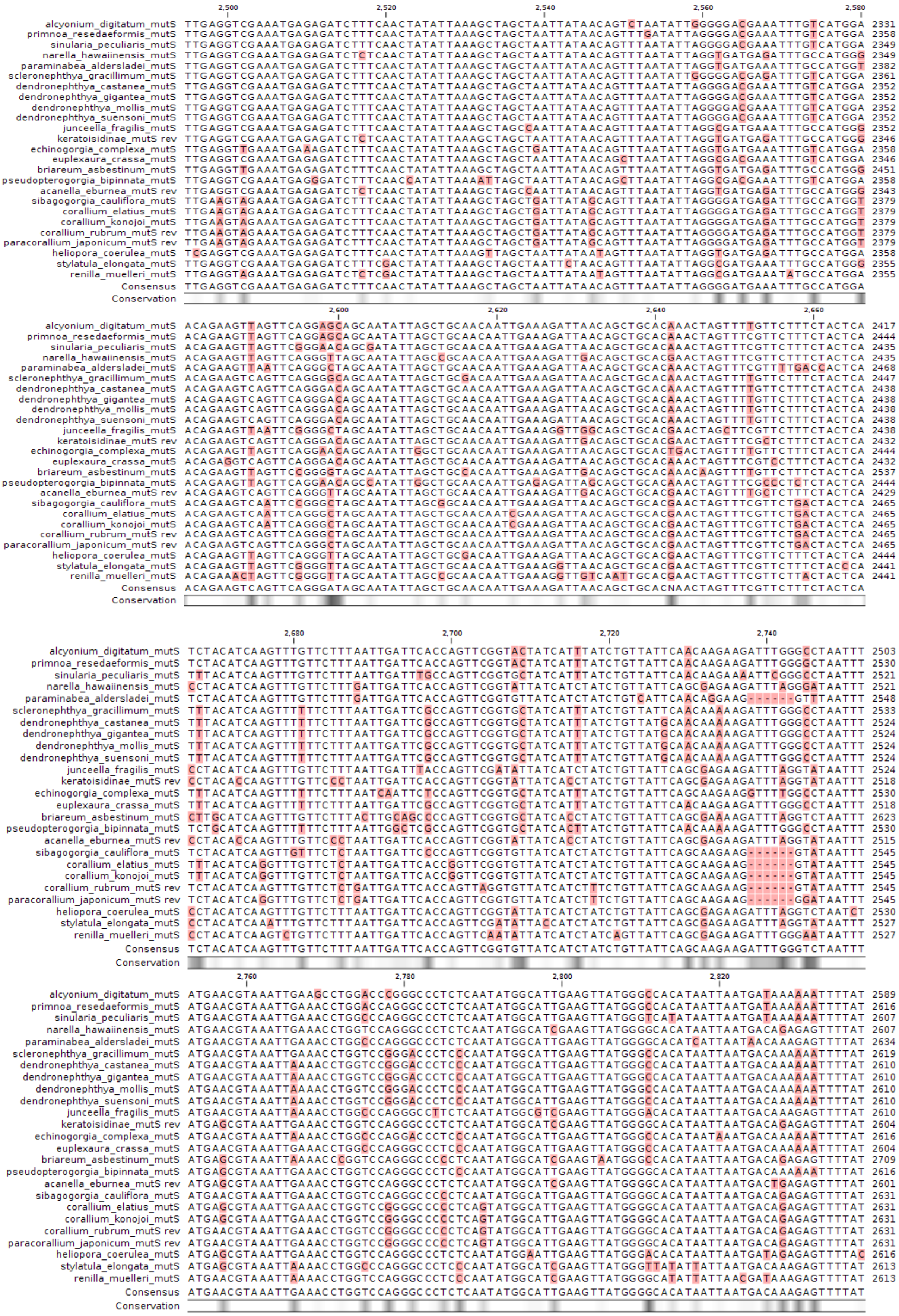
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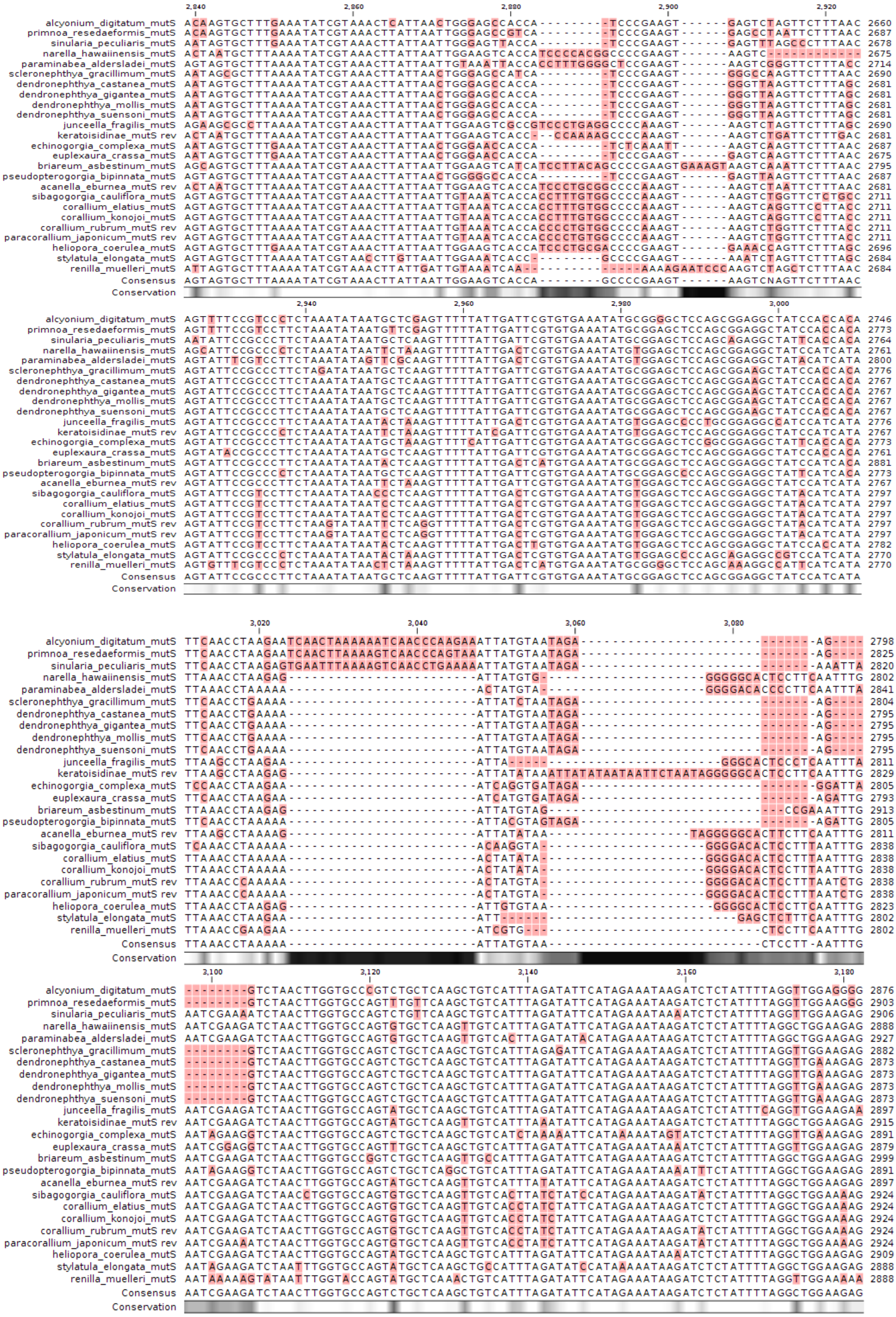
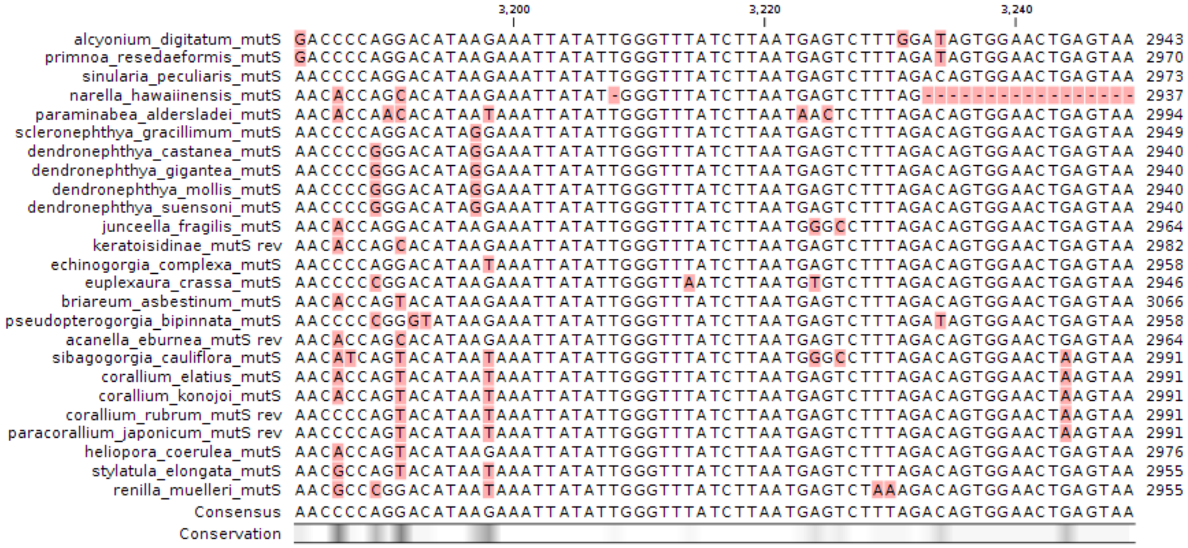
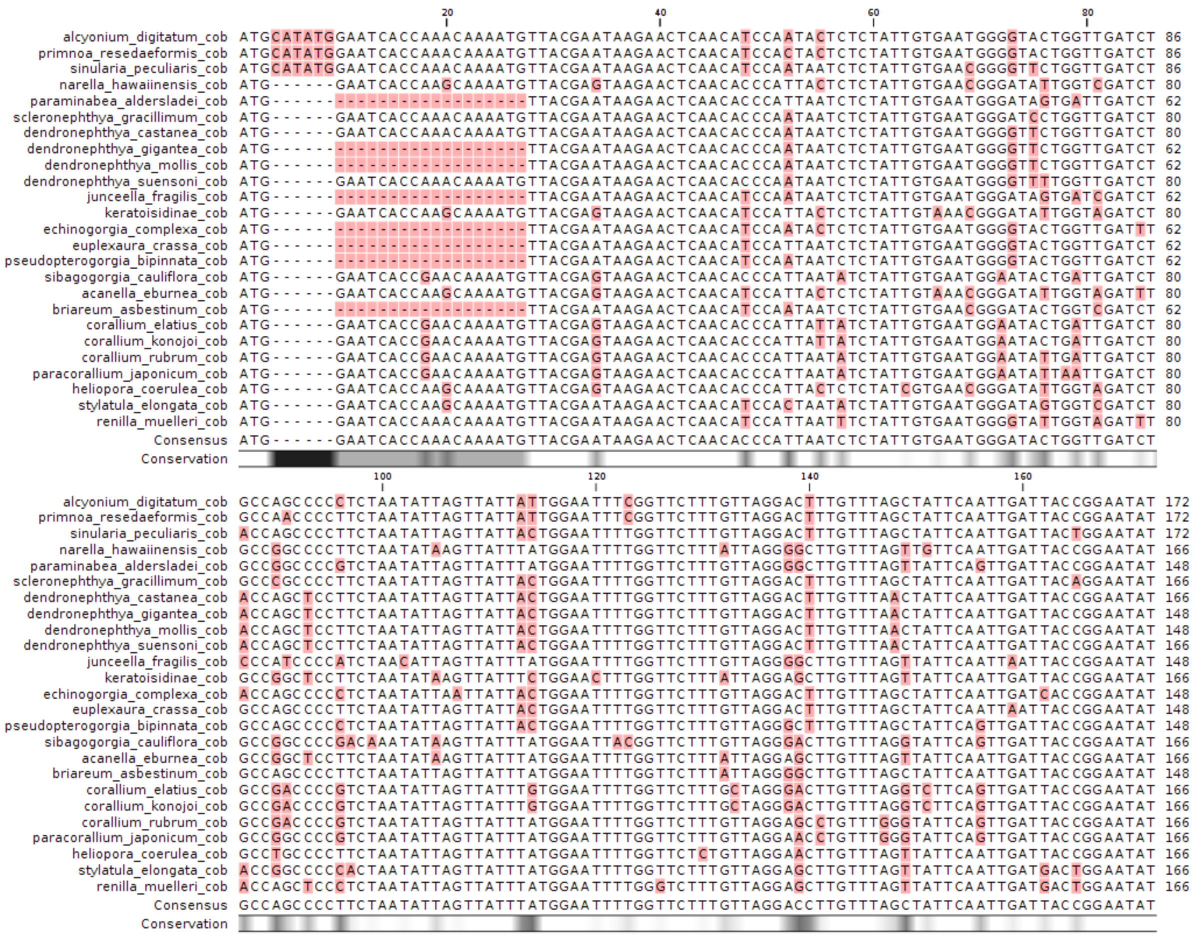
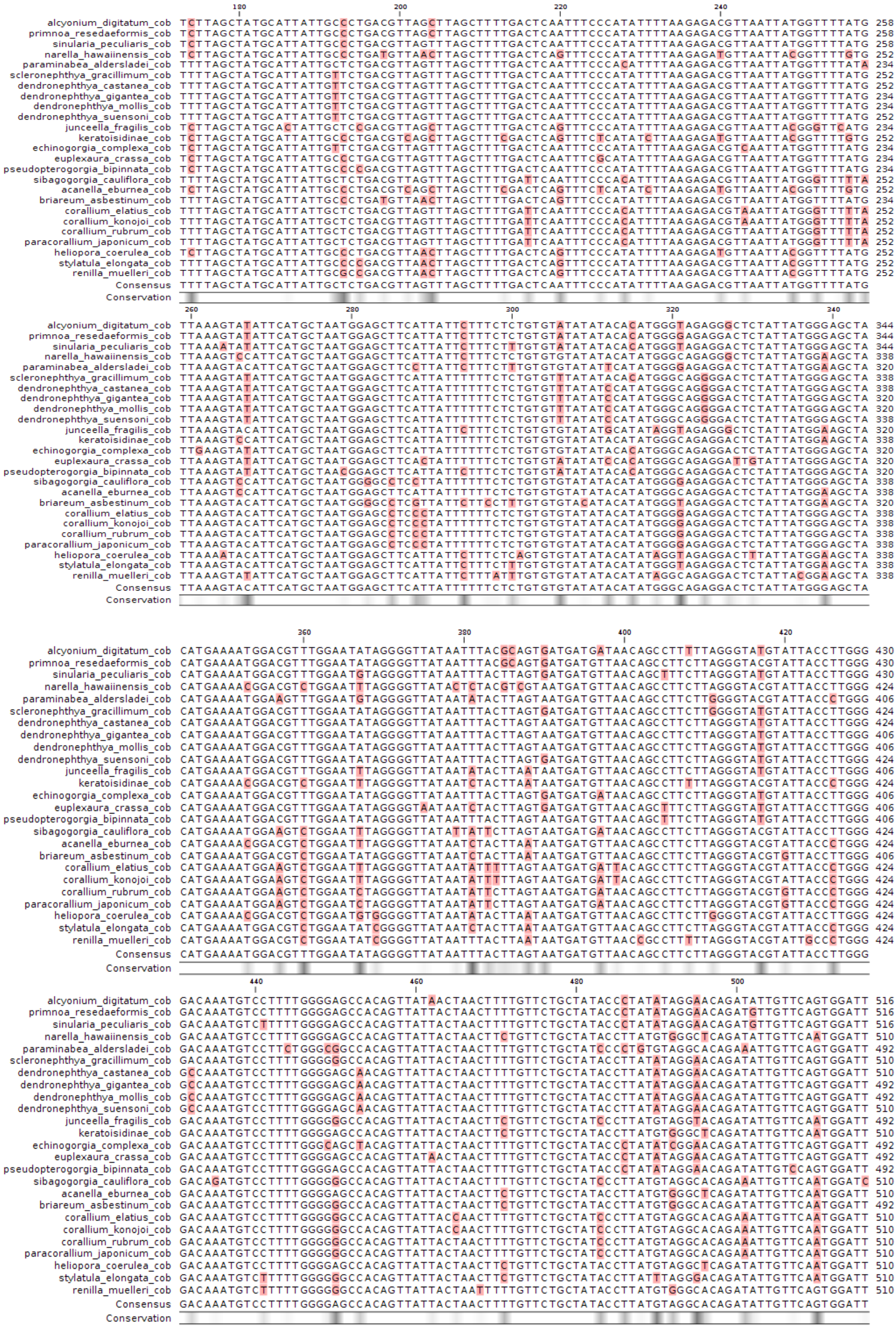
Conservation

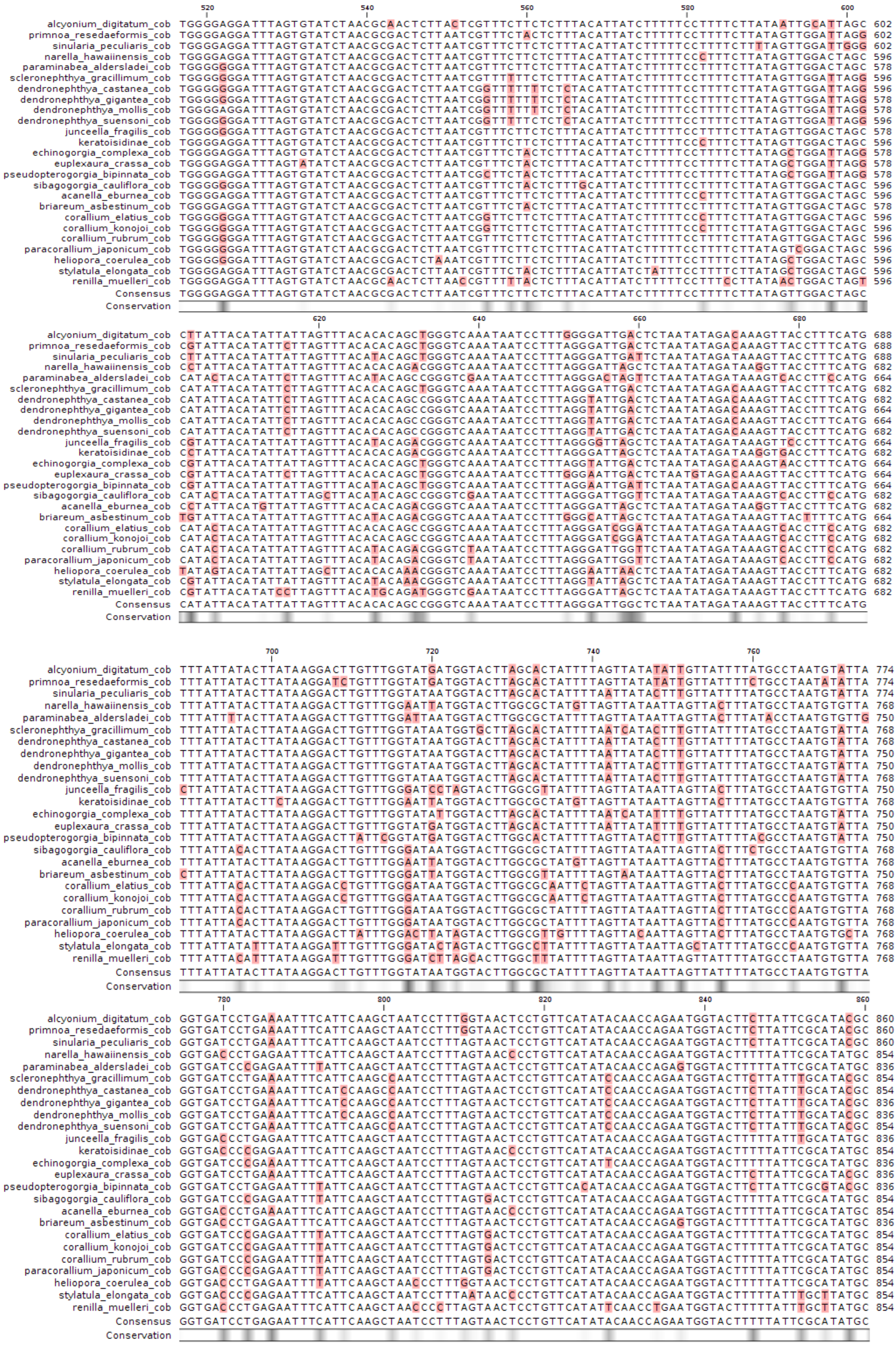


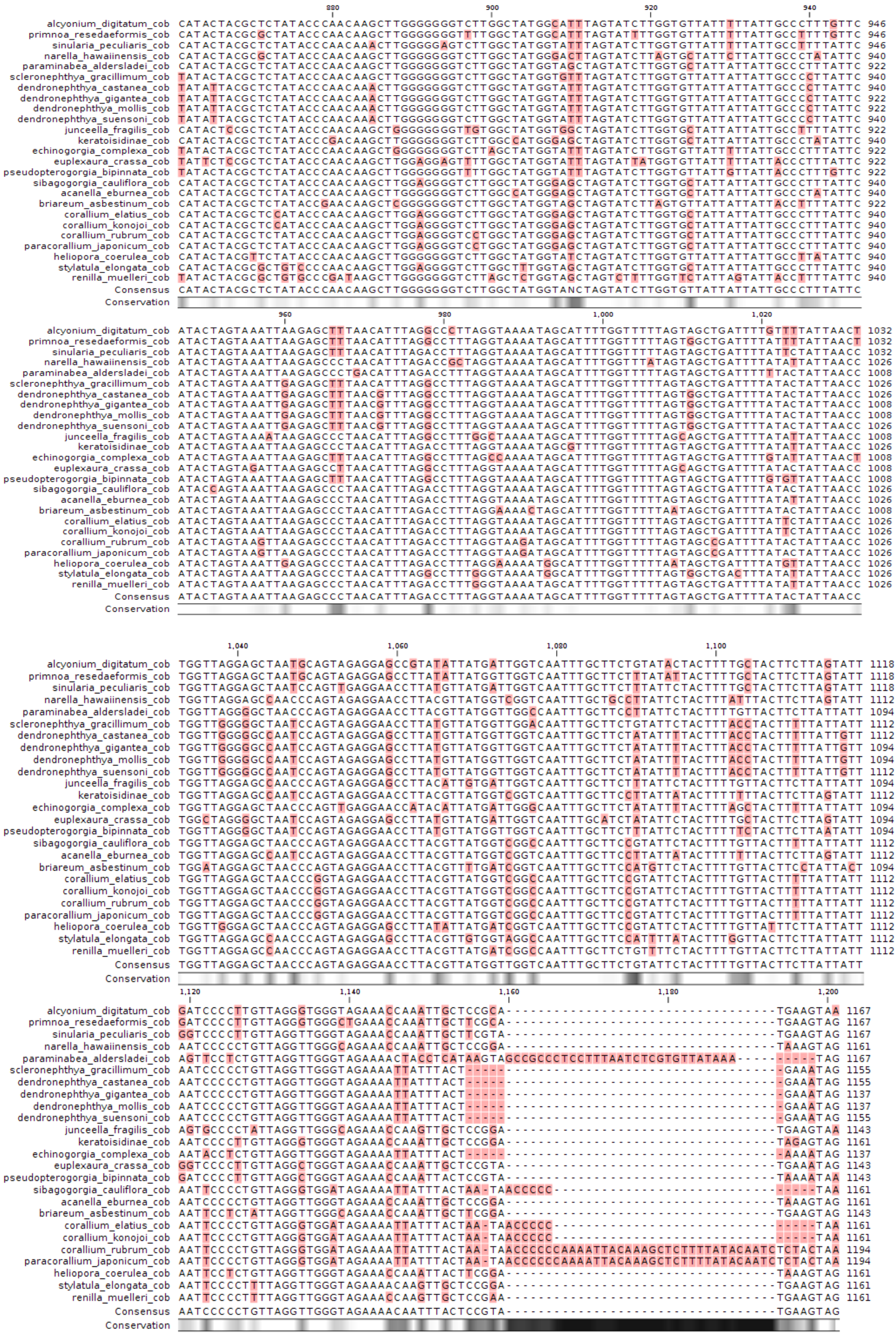


Cob gene

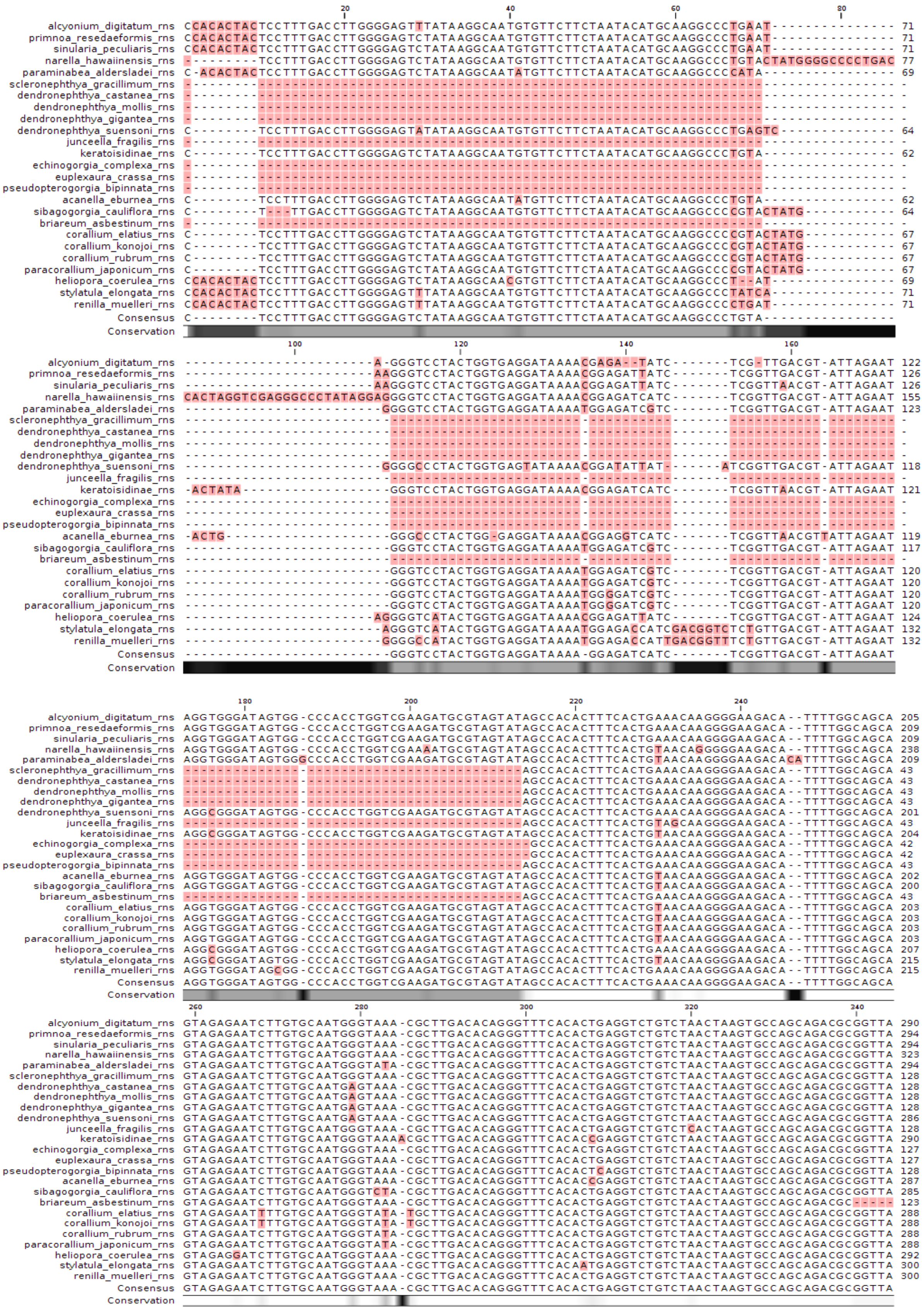


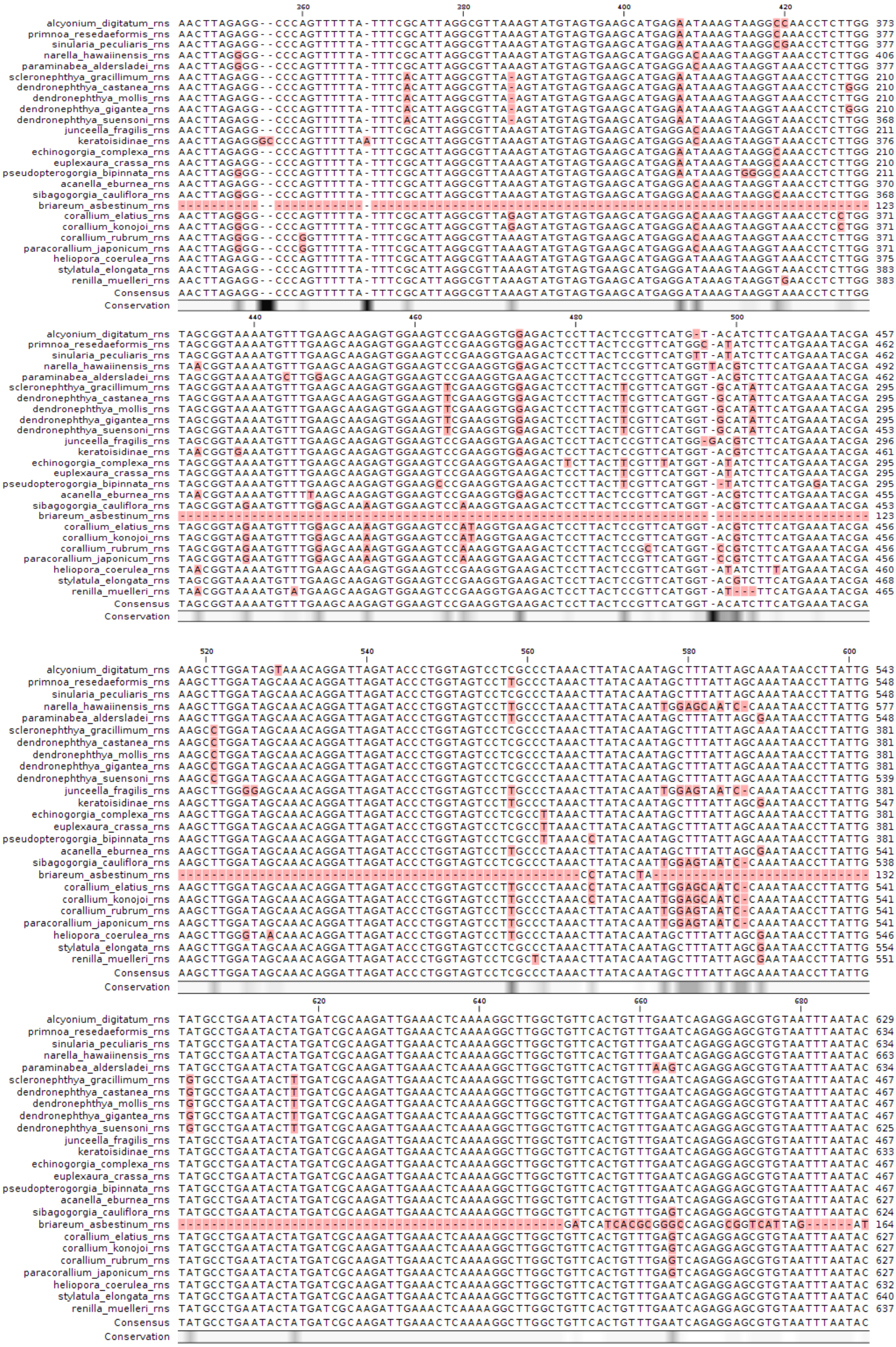






Rns gene

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400
420
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sinularia_peculiaris_rns
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scleronephthya_gracillimum_rns
dendronephthya_castanea_rns
dendronephthya_mollis_rns
dendronephthya_gigantea_rns
dendronephthya_suensoni_rns
junceella_fragilis_rns
keratoisidinae_rns
echinogorgia_complexa_rns
euplexaura_crassa_rns
pseudopterogorgia_bipinnata_rns
acanella_eburnea_rns
sibagogorgia_cauliflora_rns
briareum_asbestinum_rns
corallium_elatius_rns
corallium_konojoi_rns
corallium_rubrum_rns
paracorallium_japonicum_rns
heliopora_coerulea_rns
stylatula_elongata_rns
renilla_muelleri_rns
Consensus
Conservation
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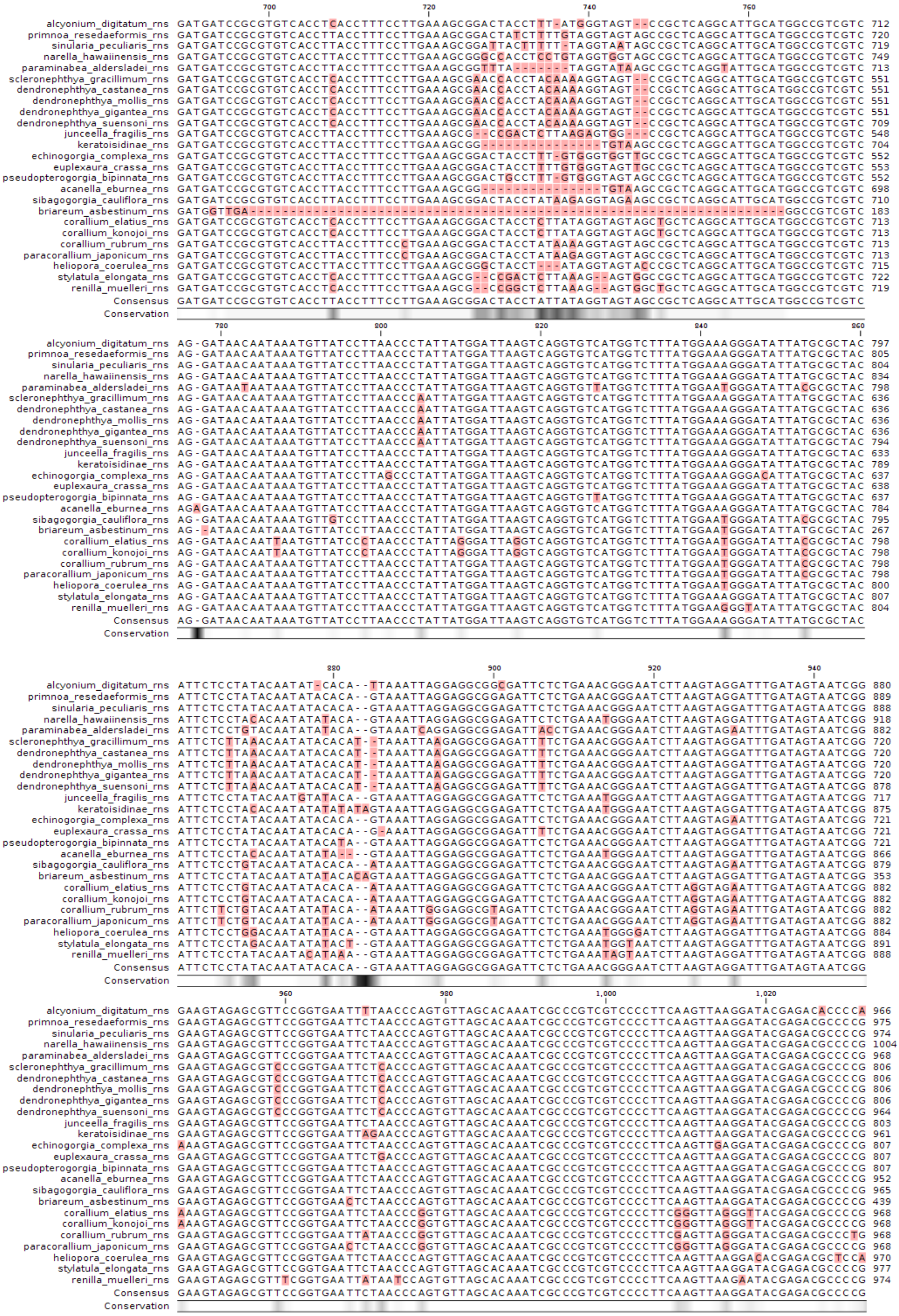
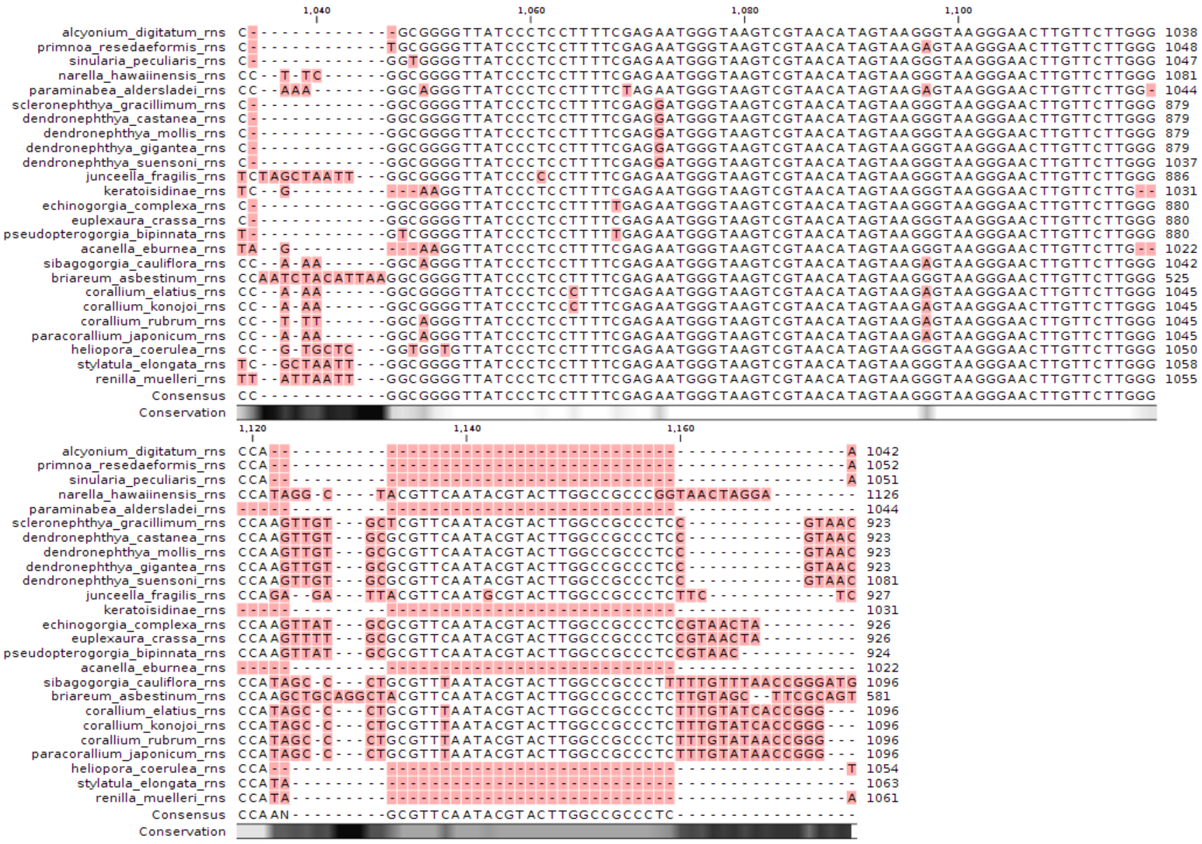
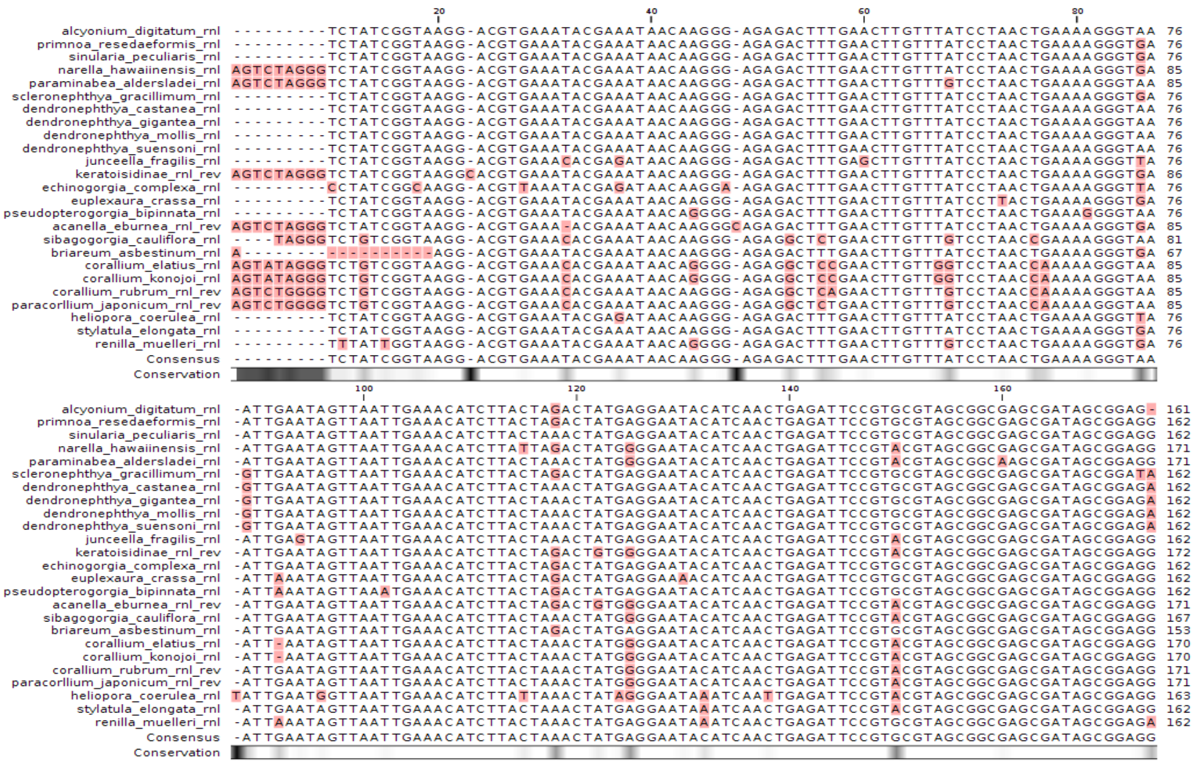
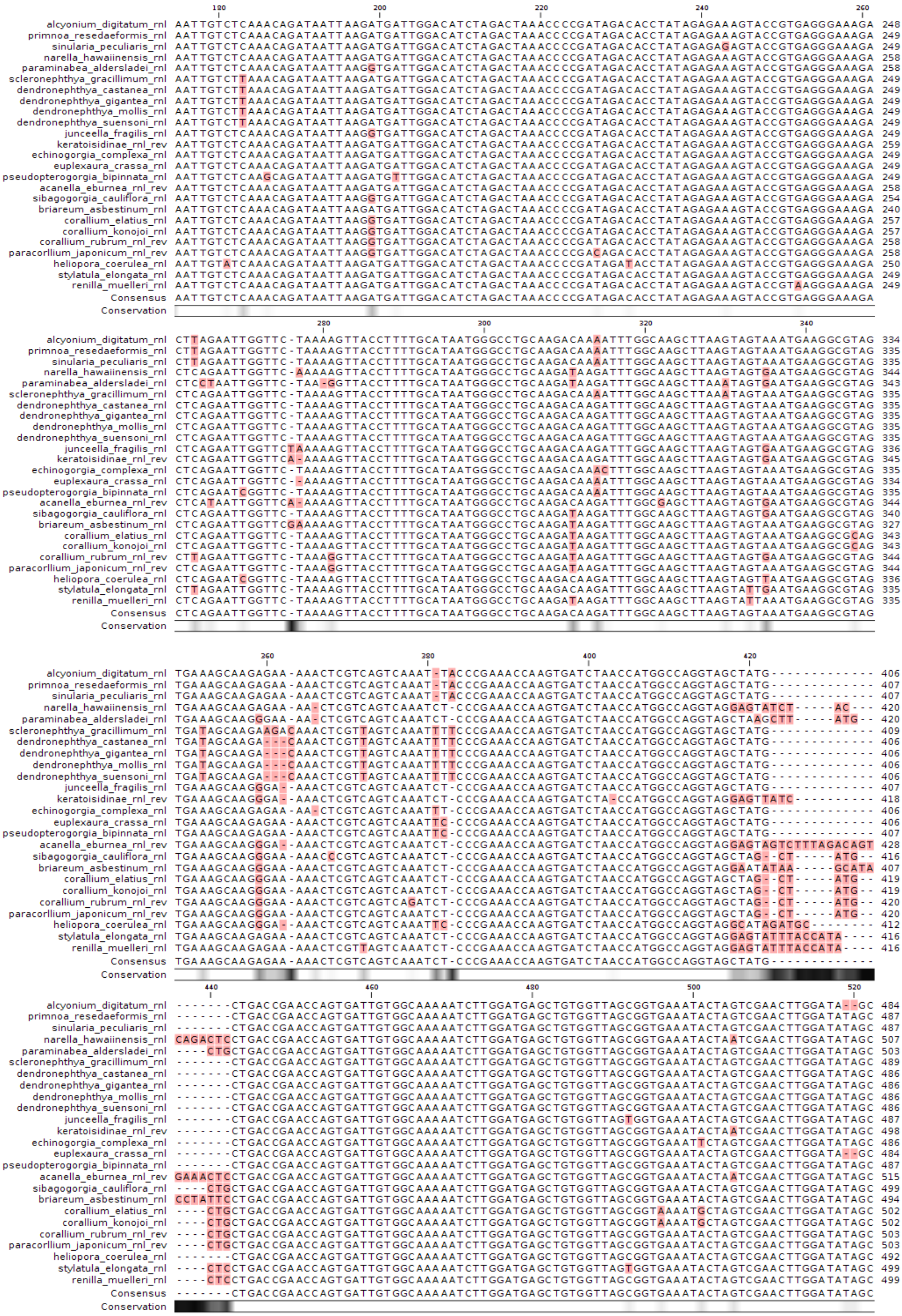
460
480
500
520
540
560
580
600
620
640
660
680





Rnl gene

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scleronephthya_gracillimum_rnl
dendronephthya_castanea_rnl
dendronephthya_gigantea_rnl
dendronephthya_mollis_rnl
dendronephthya_suensoni_rnl
junceella_fragilis_rnl
keratoisidinae_rnl_rev
echinogorgia_complexa_rnl
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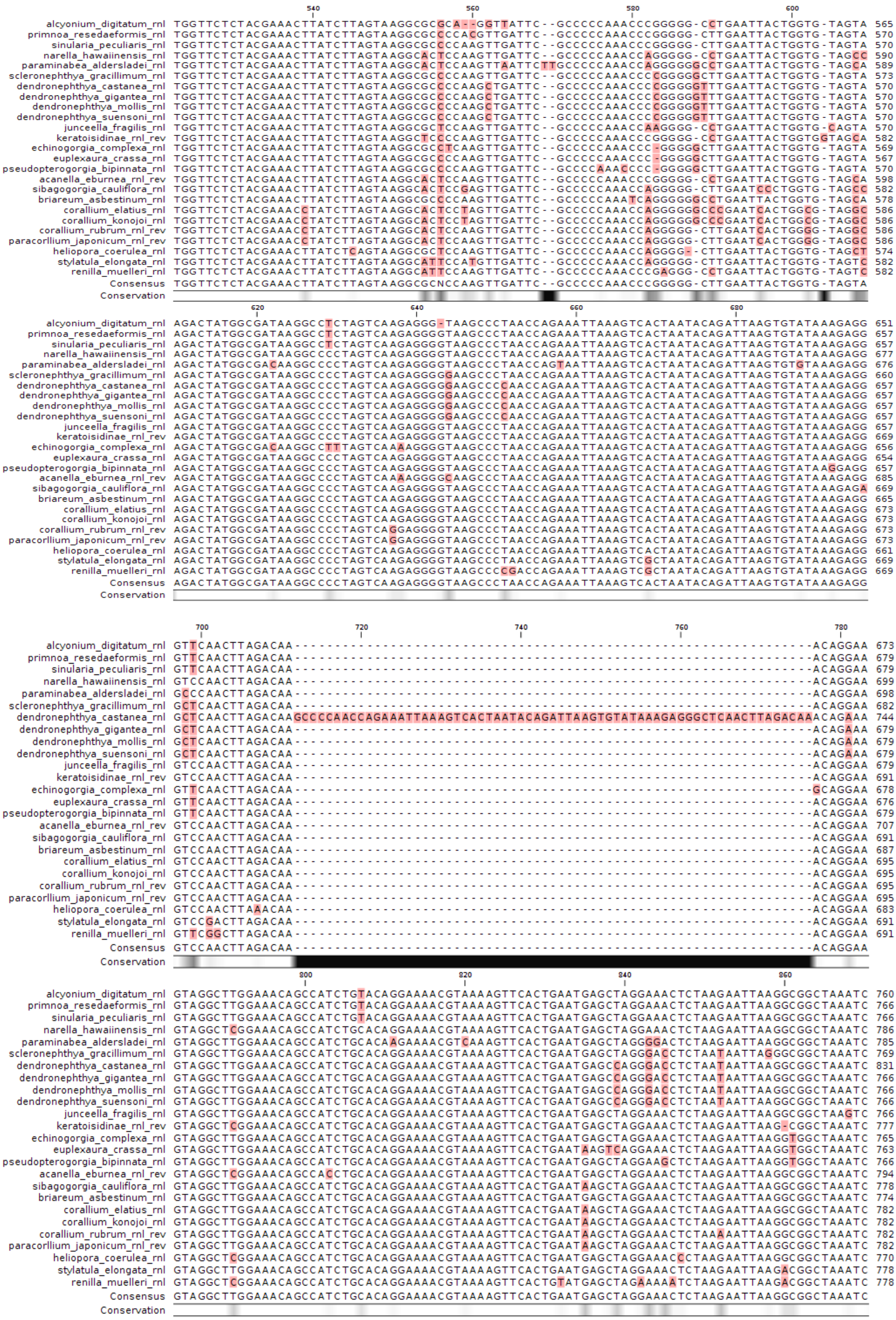
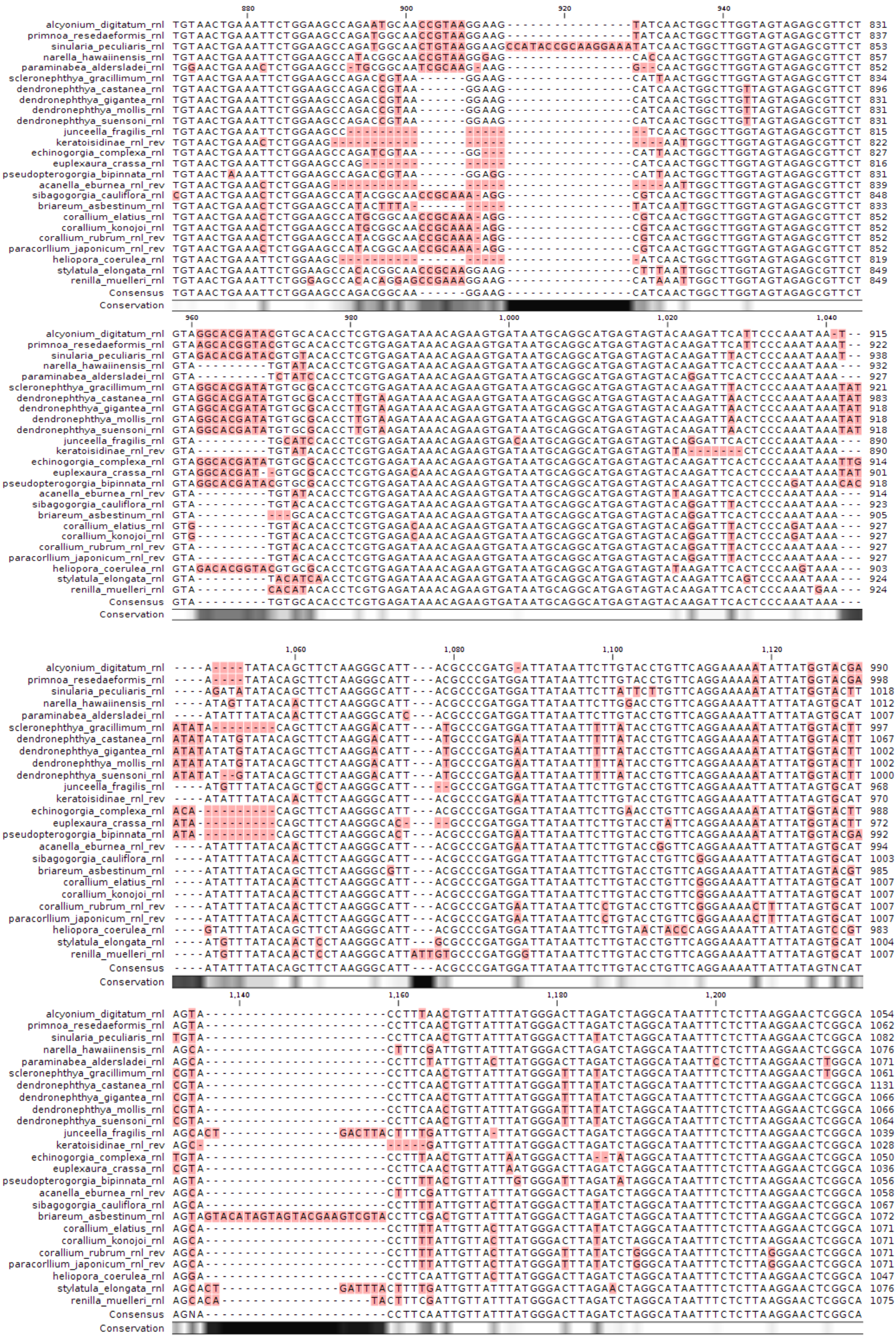
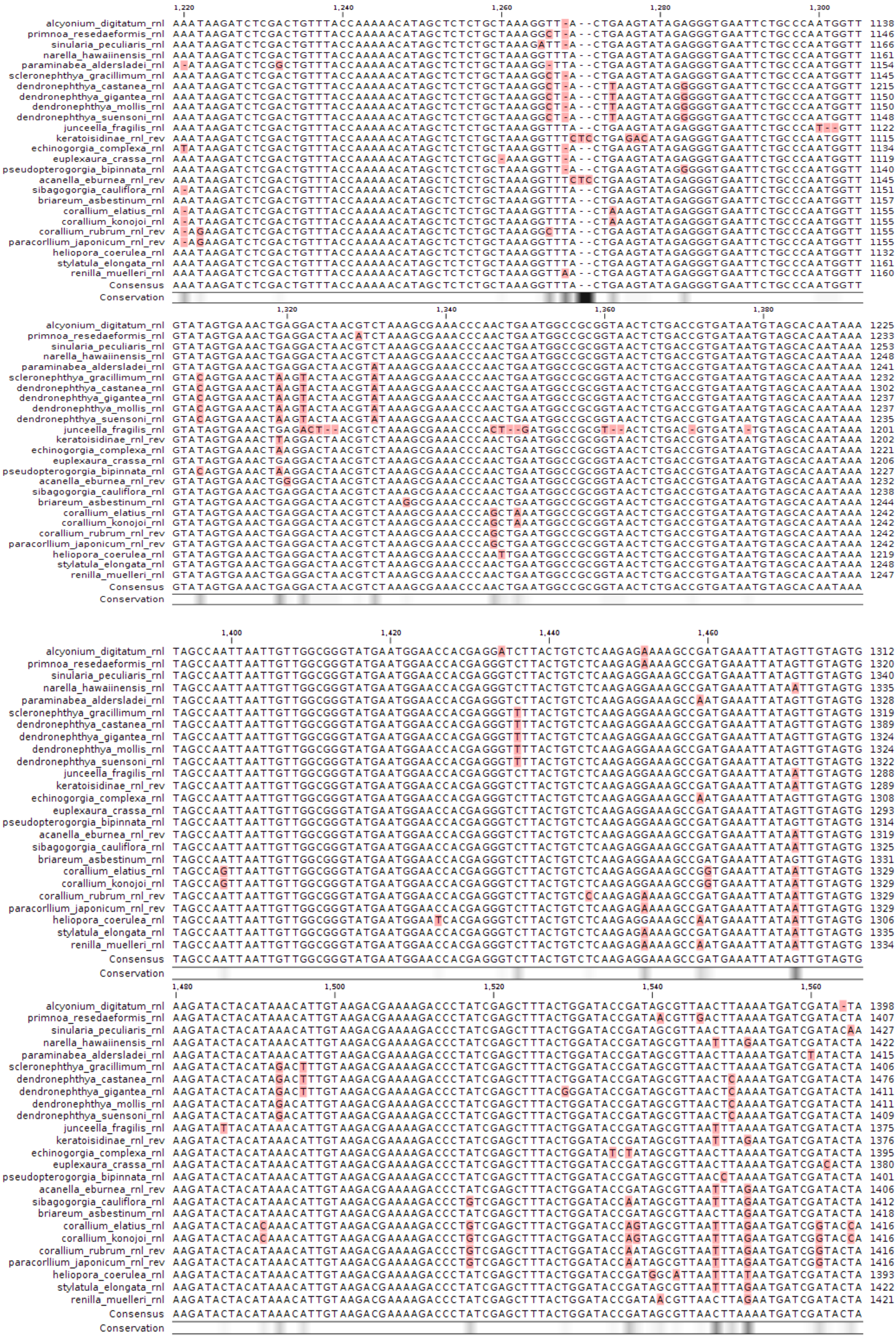
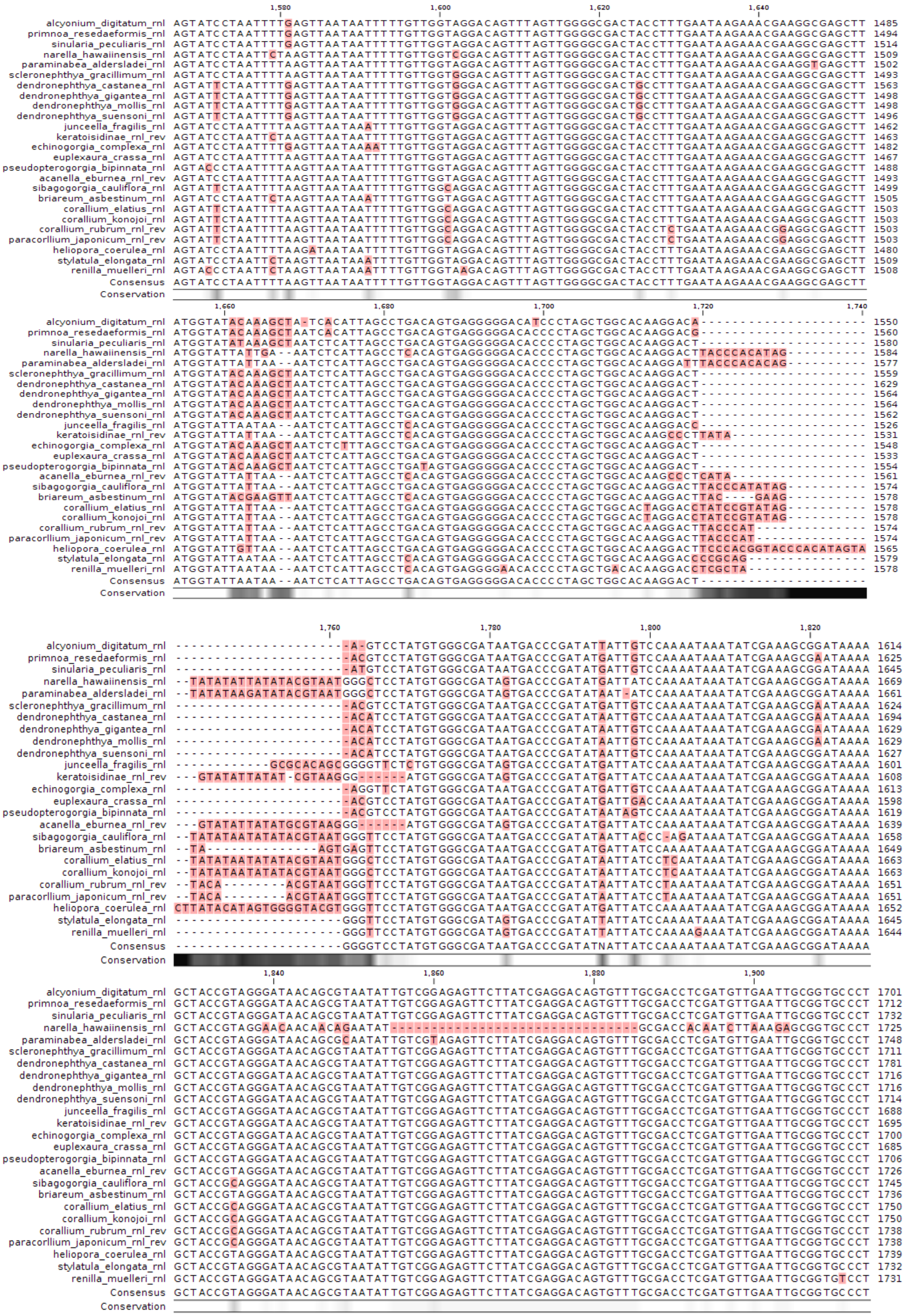
Consensus
Conservation









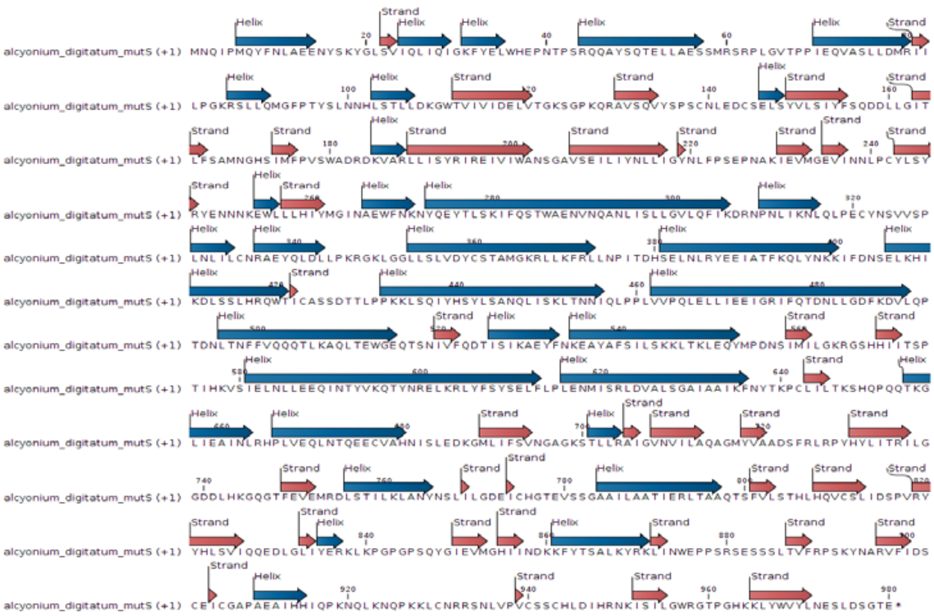
tRNA f-Met

Appendix F

Protein sequence of *mtMutS* gene in *A. digitatum*

MNQIPMQYFNLAEENYSKYGLSVIQLIQIGKFYELWHEPNTPSSQQAYSQTELLAESSMR
SRPLGVTPPIEQVASLLDMSMMLPGKSSLLQMGFPTYSLNNHLSTLLDKGWTVMVIDEL
VTGKSGPKQRAVSQVYSPSCNLEDCSELSYVLSIYFSQDDLLGITLFSAMNGHSMMFPVS
WADSDKVARLLISYRISEMVIWANSGAVSEILMYNLLIGYNLFPSEPNAKIEVMGEVMNN
LPCYLSYSYENNNKEWLLLHIYMGINAEWFNKNYQEYTLSKMFQSTWAENVNQANLISL
LGVLQFIKDRNPNLIKNLQLPECYNSVVSPLNLMLCNRAEYQLDLLPKSGKLGGLLSLVD
YCSTAMGKSLLKFSLLNPITDHSELNLRYEEIATFKQLYNKKMFDNSELKHIKDLSSLHRQ
WTMCASSDTTLPPKKLSQIYHSYLSANQLMSKLTNNIQLPPLVVPQLELLIEEMGRIFQTD
NLLGDFKDVLQPTDNLTNFFVQQQTLKAQLTEWGEQTSNIVFQDTISIKAEYFNKEAYAF
SILSKKLTKLEQYMPDNSIMMLGKSGSHHMITSPTIHKVSIELNLLEEQINTYVKQTYNRE
LKSLYFSYSELFLPLENMISSLDVALSGAIAAIKFNYTKPCLMLTKSHQPQQTKGLMEAIN
LRHPLVEQLNTQEECVAHNISLEDKGMLMFSVNGAGKSTLLSAIGVNVILAQAGMYVAA
DSFSLSPYHYLITRILGGDDLHKGQGTFEVEMSDLSTMLKLANYNSLMLGDEICHGTEVS
SGAAMLAATIESLTAAQTSFVLSTHLHQVCSLIDSPVRYYHLSVIQQEDLGLIYERKLKPG
PGPSQYGIEVMGHMINDKKFYTSALKYRKLINWEPPSRSESSSLTVFRPSKYNARVFIDSC
EMCGAPAEAIHHIQPKNQLKNQPKKLCNSSSNLVPVCSSCHLDIHSNKISILGWSGTPGHK
KLYWVYLNESLDSGTE*

Protein annotation of *mtMutS* gene in *A. digitatum*



Protein sequence of *mtMutS* gene in *P. resedaeformis*

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TGKSGPKQRAVSQVYSPSCNLEDCSELSYVLSIYFSQDDLLGITLFSVMNGHSMMFPVSW
TDSDKVARLLINYRISEMVIWANSGAGSEILMNKMYNLLIGWNLFPSEPNAKMEVMGEA
LTNLPCYLSYSYENNNKEWLLLHIYMGINAEWFNKNYQEYTLSKMFQSTWTENVNQVN
LISLLGVLQFIKDRNPNLIKNLQLPECYNSVVSPLNLMLCNRAEYQLDLLPKSGKLGGLLS
LVDYCSTAMGKSLLKFRLLNPITDHSELNLRYEEIATFKQLLDKKMFDNSELKHIKDLSSL
HRQWAMCASSDTTLPPKKLSQIYHSYLFANQLMSKLMNNKWINIQLPPSVGPQLELLIEE
MGRVFQADNLLGDFKDVLQPTDNLTNFFVQQQTLKAQLTEWAEQTSNIVFQDTISIKAE
YFNKEGYAFSILPKKLIKLEQYLTNASMSDNSIMMLGKSGSHHMITSPAIHKVSIELNLLE
EQINTYVKQTYNRELKSLYFSYSELFLPLVNMISSLDVALSGAIAAIKFNYIKPCLMLAKS
QQTKGLMEAINLRHPLVEQLNTQEECVAHNISLEDKGMLMFSVNGAGKSTLLSAIGVNVI
LAQAGMYVAADSFSLSPYHYLITRILGGDDLHKGQGTFEVEMSDLSTMLKLANYNSLML
GDEICHGTEVSSGAAMLAATIESLTAAQTSFVLSTHLHQVCSLIDSPVRYYHLSVIQQEDL
GLIYERKLKPGPGPSQYGIEVMGHMINDKKFYTSALKYRKLINWEPSSRSEPNSLTVFRPS
KYNVRVFIDSCEMCGAPAEAIHHIQPKNQLKSQPSKLCNSSSNLVPVCSSCHLDIHSNKISI
LGWKGTPGHKKLYWVYLNESLDSGTE*

Protein annotation of *mtMutS* gene in *P. resedaeformis*