DEVELOPMENT OF A QUICK MOLECULAR BASED TECHNIQUE FOR IDENTIFICATION OF ZOOPLANKTON IN THE TURKISH COAST OF BLACK SEA

by

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DEVELOPMENT OF A QUICK MOLECULAR BASED TECHNIQUE FOR IDENTIFICATION OF ZOOPLANKTON IN THE TURKISH COAST OF BLACK SEA

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ABSTRACT

A new species identification method is introduced in this thesis. For this method, Zooplankton and fish species from the Turkish coast of Black Sea are used as a dataset. The method uses High Resolution Melting (HRM) curves to identify species. Zooplankton are essential for marine ecosystems. These organisms form a middle ground in the food chain, which sustain the energy flow between producers and consumers. Therefore, as a candidate, the most suited organism group for monitoring the Black Sea marine ecosystem is Zooplankton. However, identifying the Zooplankton species with conventional morphological methods needs time, expertise and effort. DNA barcoding can be considered as a good alternative for conventional morphology-based identification. On the other hand, it is time consuming, expensive and also limited to the information recorded in the universal DNA databases. The goals of this study were to identify Zooplankton species in Black Sea and develop a new identification methodology which is not only cost and time effective but also do not require expertise. Expediently, new software created which could use the HRM curves as a specific signature to identify or categorize the samples according to their species. In terms of high grouping capacity, this study represents a first comparison of melt curves with a specific database. The results indicate that HRM based identification is working and quite feasible. The created HRM software is managed to identify the samples as predicted in our hypothesis.

ÖZET

Bu tezde yeni bir tür tayini yöntemi geliştirilmiştir. Bu yöntem için Karadeniz'in Türkiye kıyılarından alınan balık ve Zooplankton organizmaları kullanılmıştır. Yöntem Yüksek Çözünürlüklü Erime (HRM) eğrilerini kullanarak türleri ayırt etmektedir. Karadenizde bir çok türün neslinin istilacı türler ve beşeri etkenlerden dolayı tükenme eşiğine geldiği bilinmektedir. Bu durum, Karadenizdeki ekosistemin sağlığını etkileyerek, hem ekonomik anlamda hemde doğal biyolojik mirasımızın kaybı konusunda endişe yaratmaktadır. Bu sebeple, Karadenizin sürekli kontrol edilerek, türlerin belirlenmesi gerekmektedir. Zooplankton denizdeki yaşam için son derece önemli organizmalardır. Beslenme zincirinde ara basamağı oluşturarak üreticilerle tüketiciler arasındaki enerji akışını sağlamaktadırlar. Bu nedenle Karadenizin ekosistemini gözlemlemek için en uygun canlı grubu Zooplankton türleridir. Fakat, Zooplankton türlerinin morfolojik yöntemlerle belirlenmesi uzmanlık, emek ve zaman gerektiren bir süreçtir. Morfolojik yöntemin alternatifi olarak kullanılabilecek olan DNA barkodlama ise görece pahallı ve zaman alan bir metot olup, ilgili veritabanındaki bilgilerle sınırlıdır. Bu çalışmadaki amaç Karadenizdeki Zooplankton türlerini belirlemek ve daha hızlı, ekonomik, uzmanlık gerektirmeyen yeni bir uygulama geliştirmektir. Yüksek çözünürlüklü erime eğrilerini kullanabilen bir yazılım oluşturularak, bu eğrilerin türleri ayıran bir çeşit dijital imza gibi kullanılması hedeflenmiştir. Erime eğrisi verilerini bu şekilde çok yüksek sayılarda gruplandırabilmesi ve bir veribankası ile karşılaştırması yönünden bu çalışma bir ilki teşkil etmektedir. Sonuçlar, HRM yazılımının çalıştığını ve gerçekten uygulanabilir olduğunu göstermiştir. Bu yeni yaklaşımın bazı yazılımsal ve deneysel eksikliklerin çözülmesi durumunda Tür tanımlaması konusunda geniş çaplı bir etki yaratması olasıdır. HRM yazılımı ile Zooplankton dışında da birçok farklı organizma grubu tanımlanabilecektir.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS/ABBREVIATIONS

Symbol	Explanation
COI	Cyctochrome Oxidase c Subunit I
DNA	Deoxyribonucleic acid
ddNTP	Dideoxynucleotide triphosphates
dNTP	deoxynucleoside triphosphate
HRM	High Resolution Melting
gDNA	genomic DNA
ITS	internal transcribed spacer
matK	chloroplast maturase K
mtDNA	mitochondrial DNA
PCR	Polymerase Chain Reaction
QPCR	Quantitative Polymerase Chain Reaction
rbcL	ribulose-bisphosphate carboxylase
Tm	Melting Temperature

1. INTRODUCTION

In Turkey, a number of species have extinct while survival of many other species is in danger due to the effects of increasing water pollution, the presence of invasive species and other anthropogenic impacts. By taxonomists, almost 600 invasive macrophyte, invertebrate and fish species have been described to be present, mainly in the coastal habitats of the Mediterranean (Galil, 2007). These invasive species have reached a region of the Black Sea, where their effects upon ecosystem of that area are known to be quite destructive. However, such information on this subject is rather limited since very few studies have investigated the effects of invasive species on the natural biota of the Black Sea. As their numbers rise, there is an increasing possibility that the presence of some invaders remains as yet unknown and it is thought that these species could cause major changes to the biological diversity of the area (Kideys, 2002).

It is extremely difficult to identify marine Zooplankton using classical morphological methods, which require expertise and intensive effort (Pichera et al., 2004). This situation harms our understanding of ecosystem dynamics by making harder routine monitoring programs. In biodiversity studies, the classical taxonomical system used for many years has been seen as the most reliable technique for classification. However, molecular-based studies conducted in the last 30 years, have shown that some species previously accepted as the same, actually different from one another (Tautz et al., 2003). It was proposed that using a single target gene (mitochondrial cytochrome oxidase subunit 1 - COI) for the separation of animal species (Hebert et al., 2003). Subsequent studies demonstrated that COI-targeted DNA coding could be used to differentiate species in both vertebrate and invertebrate animals (Weigt et al., 2012; Francis et al., 2010; Hubert et al, 2008).

DNA Barcoding studies comprise the polymerase chain reaction (PCR), sequencing, and phylogenetic analysis, respectively (Hubert et al., 2008). With this

method, identification of an organism can be completed in approximately 3 days. Although it is possible to obtain the same result with next-generation sequencing (NGS) within hours, the high-cost of NGS analysis restricts its use for routine monitoring studies (Ebenezer et al., 2012). The lack of comprehensive molecular-based studies on Zooplankton diversity in Turkish coastal waters is caused by several factors. These are the high analysis costs, the time required to carry out DNA barcode analysis and the need for expertise in the field of molecular genetics. The high cost and duration of DNA Barcoding can be reduced by using the technique of high-resolution melting analysis (HRM) which can produce results of similar sensitivity and accuracy (Montgomery et al., 2007). The HRM analysis has emerged following a combination of HRM dyes with simultaneous DNA duplication in QPCR. HRM can be completed within a 5-10 min period of post-PCR analysis generating different HRM profiles based on different DNA sequences. Therefore, it is possible to analyze and categorize hundreds of DNA sequences in a short time. Despite the wide use of HRM analysis in many different viral and microbial studies (Dhakal et al., 2013; Gago et al., 2013; Jin et al., 2012), this method has not yet been used in eukaryotic diversity studies (Calvès et al., 2013; Brechon et al, 2013). With the proposed project, we aim to reduce the time, cost and effort for species identification by creating a unique database of HRM profiles which specific to Zooplankton species in the Black Sea. In this context, Zooplankton organisms were collected from Turkish coasts of Black Sea. The species of the samples were morphologically identified by Ms. İlayda Destan Öztürk. Then the mtDNAs of the samples were extracted. PCR experiments were carried out in order to amplify the COI gene of the samples. These products were melted to obtain HRM profiles and then sequenced to molecular identification. A database constructed from HRM profiles, which were belong to morphologically and molecularly identified organisms. Thus, a software was constructed to analyze and compare unknown HRM profiles with this database for quick identification.

1.1. An Overview of Zooplankton

Zooplankton are a general name for animals drifting in the water. Most of them have a small capacity to swim against the water movements. Zooplankton are irreplaceable in marine ecosystem in the aspects of taxonomic composition, morphological variation and

food chain sustainability. They are composed from a broad range of organisms, including unicellular organisms to vertebrates (Harris et al, 2000).

Classification of Zooplankton is made by their size, habitat, taxonomic position or developmental progress. Zooplankton can be divided into two main groups as holoplankton and meroplankton. Holoplankton stay in planktonic stages during their whole life cycle, such as Calanoid copepods. On the other hand, members of meroplankton stay in planktonic stages in a part of their life cycle like fish larvae (Al-Yamani et al., 2011). There are 3 main categories for identifying Zooplankton according to their size, which are Macrozooplankton, Mesozooplankton and Microzooplankton as indicated in table 1.1. (Van Couwelaar et al., 2003)

Table 1.1. Main categories of Zooplankton types according to their sizes.

Zooplankton Type	**Size**	**Organisms**
Macrozooplankton	$2x10^4$ - $2x10^7$ μm	Salps, Doliolids, adult and juvenile Decapods
Mesozooplankton	200-$2x10^4$ μm	Copepods; Cladocerans; Ostracods; Chaetognaths
Microzooplankton	20 - 200 μm	Foraminiferans; Radiolarians; Ciliates, Rotifers

Zooplankton have a significant effect on the marine ecosystem. They feed on phytoplankton and they are food for fish species. This makes them a keystone in the marine food chain. Therefore, Zooplankton are responsible from a lot of pelagic phenomenon such as dynamics of fish stocks or control of spring bloom (Dagg et al., 2008). In addition to ecological aspects of Zooplankton, using them to measure pollution level and characterize the pollution types are also very important for marine systems because pollution is one of the most dangerous threat to the marine environment (Gajbhiyeet al., 2002). Moreover, monitoring the changes in Zooplankton dynamics is used

for measurement of global ecosystem dynamics and biogeochemical cycles (Buesseler et al., 2001).

The Black Sea Large Marine Ecosystem (LME) is almost isolated from other marine system. The Bosphorus and Dardanelles straits are the only channels to connect Black Sea LME to the oceans through the Marmara and Mediterranean seas respectively. The Black Sea LME and Sea of Azov together consist from nearly 460,150 km^2 (Heileman et al., 2008).

The Black Sea LME has an excessive ecosystem production rate which is over than 300g/cm^2 per year. This excessive primary production rate is related to the river streams which are fed the Black Sea (Sur et al., 1994), also related to the natural primary production in winter (Nezlin et al., 1999)**.**

The fishing industry is an important economical income for the countries bordering the Black Sea LME. Almost all the fish stocks in the Black Sea LME are collected from these countries (FAO, 2014).

Extreme and illegal fishing have adversely affected the fish stocks in the Black Sea LME. Especially, introducing of the new fishing technologies like purse seining and gill net are responsible from enormous diminish in the valuable fish populations such as mackerel, bonitoa, horse mackerel and anchovy (Caddy, 1993). Besides the overfishing, the other main reason which responsible for decreasing in fish stocks is the increment in eutrophication level (Daskalov, 2003).

Apart from these reasons, invasive species could also affect the fish stocks by disturbing the balance of the Black Sea LME. For instance, *Mnemiopsis leidyi* which is an invader ctenophore is detected in Black Sea in1980s (Vingradov et al, 1989).This organism

was considered as the reason for decreasing of the fish stocks because it consumes the food of the fish species (Kideys, 1994).

1.2. Studying Zooplankton

The Zooplankton sampling is managed by collection, fixation and preserving steps. Zooplankton collection methods are based on the filtration of water by various tools. These are mainly the bottles, the pumps and the nets (Goswami, 2004).

In Bottle method, the water is captured in a sterile bottle which is usually having 5 to 20 liter volume. Therefore, this method is useful for capturing the Microzooplankton rather than the bigger forms of Zooplankton. This method is easy to operate and it allows getting exact information about sampling depths. However, this method is limited when there is a need to collect bigger Zooplankton forms, because of the relatively small amount of water is filtered (Goswami, 2004).

In pump method, the water body is continuously sucked by a pump and Zooplankton organisms are separated by a filter. Inlet pipe of the pump is set in the water and outlet pipe is set in the filter. A volume counter records the water amount which is passed through the filter. Therefore, this method is efficient for quantitative analysis of Zooplankton distribution, also for continuous experiments. The disadvantage is that the bigger Zooplankton members like jellies can be disrupted by the filter or friction force. Besides, the sampling depth is restricted by a few meters and it is not convenient to get deep layer samples (De Bernardi, 1984).

The most preferred method for Zooplankton sampling is collection using the net systems. The reasons for that net systems are allow the quantitative and qualitative measurements when a suitable gear is used by the net. Besides, it allows the sampling from deeper layers of the water and excessive amount of water (De Bernardi, 1984).

Generally, the plankton net is in conical shape and consists of a filtering cone and collecting bucket. Depend on the mesh size of the net, different type of Zooplankton forms can be captured. Moreover, Zooplankton can be collected either vertically or horizontally in the water body. These parameters make the net system more attractive than the other collection methods (Goswami, 2004).

It is a common sense that fixation of a sample is crucial for the further analysis steps. Fixation of Zooplankton samples is usually handled in 5 minutes after sample collection in order to avoid any deterioration. The most common fixative for Zooplankton is formaldehyde (4-5%) because it is cheap, non-corrosive and it kills the organisms very quickly. Besides the formaldehyde, ethanol, picric acid and acetic acid are also used for fixating the Zooplankton organisms. The optimal solvent for fixation solution is the local water of the Zooplankton themselves because the osmotic pressure adjustment would be unnecessary (Gifford et al., 2000).

After fixation, it is very important to preserve the samples until the research aim is fulfilled. The Zooplankton samples are transferred to an air proof container with enough preservative solution. The preservatives could be buffered formalin (4-5%), 70% ethanol or 40% isopropanol solutions depend on the aim. In addition, the temperature of the preserved samples is important, samples must be stored at less than 25^0C (Goswami, 2004).

1.1.1. Taxonomic Identification Of Zooplankton

Species identification is very important to get knowledge about distribution pattern, seasonal variability and community structure of Zooplankton in an aquatic ecosystem. Taxonomic identification of Zooplankton requires a sufficient amount of time, expertise, and published data. Taxonomists use the checklists as shown in the figures 1.1, 1.2 and 1.3

as examples to identify common groups in the first place. Then, they identify the species with their expertise and experience (Montagnes et al., 1993).

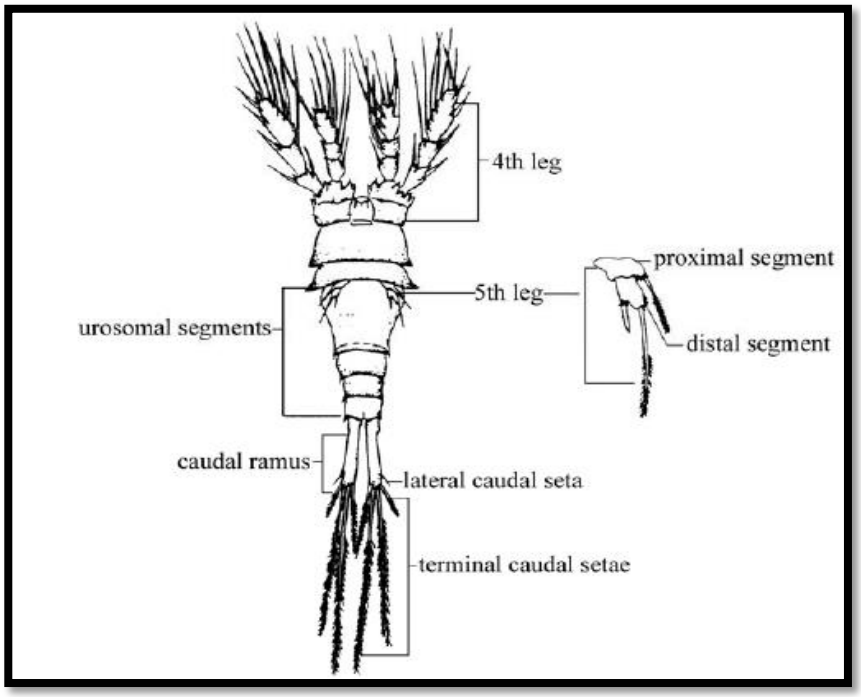

Figure 1.1. Ventral view of posterior segments and appendages in a typical cyclopoid copepod showing key identification structures (Hudson et al., 1988).

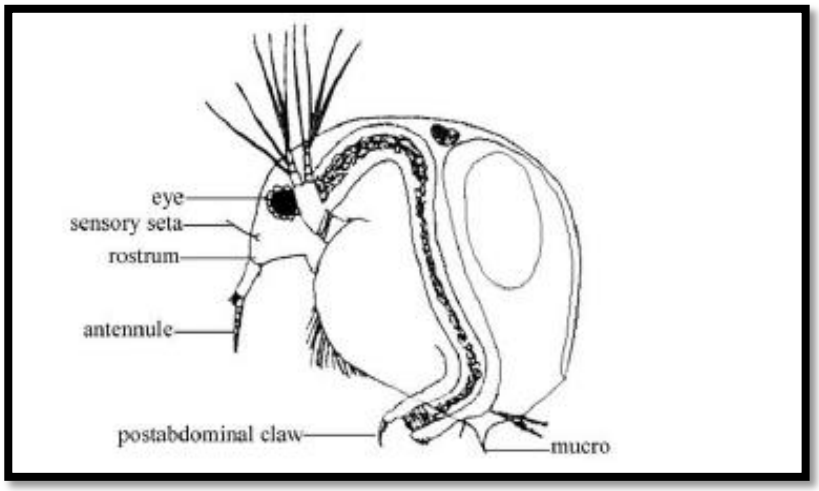

Figure 1.2. General diagram of a member of the family Bosminidae (Pennak, 1989).

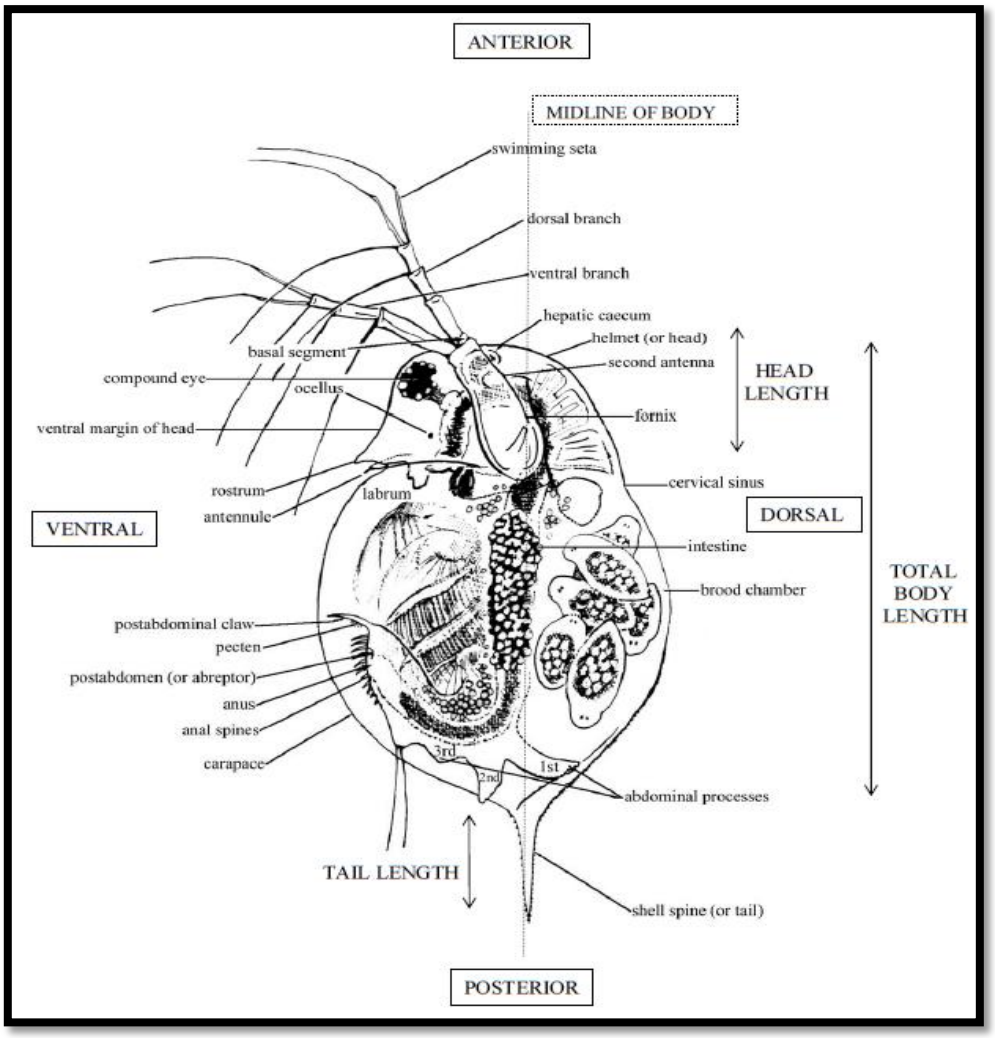

Figure 1.3. General anatomy of a cladoceran (Dodson et al., 1991).

1.1.2. DNA Barcoding

DNA Barcoding is a species identification system that firstly proposed by the research group of Paul Heber in 2003. According to Hebert, specific DNA sequence from a standardized part of the genome can be used to differentiate organisms in species level (Hebert et al., 2003). This DNA sequence is called as a barcode and for animal species generally Cytochrome C Oxidase 1 (COI) gene used for this purpose. Using COI gene as a barcode has many advantages. First, it is located on mitochondrial genome; hence each cell has a number of copies of COI gene. Second, a short sequence of COI as nearly 658 base pairs is sufficient to discriminate most of the animal species (Hubert et al., 2008). Moreover, it is easy to amplify and it is cheaper to sequence because of the being short.

DNA is located in the nucleus of Eukaryotic cells and into the cytoplasm of prokaryotic cells. In addition, some organelles like mitochondria and chloroplast also have their own DNA molecule which is located inside of these organelles (Russell, 2001). In order to extract the DNA from a cell or organelle, the cells must be disrupted by physical or chemical methods depend on the cell type and the expected DNA quality. Then the unwanted pieces of the cell must be washed away like cell membrane, RNAs and proteins. Finally, the eluted DNA must be stored in a proper solution, which has the convenient pH values (Butler, 2011).

In order to obtain COI sequence from the mixture of genomic and mitochondrial DNA, polymerase chain reaction (PCR) is performed with the pre-designed primers. PCR is an enzymatic reaction that produces millions of copies of specific sequences between two segments of template DNA. The wanted region of the DNA is determined by using the two short oligonucleotides called primers which are complementary sequences for both sense and antisense strand of the DNA. When the template DNA is heated enough to be single stranded, temperature is decreased to let the primers bind their complementary sequences on the DNA. Then the temperature is adjusted to the optimum for a DNA polymerase enzyme which synthesizes the new strand by using the single strand DNA as the template. Therefore, this cycle is carried out for 30-50 times depend on the reaction quality. After each of these cycles, the number of the specific DNA sequence would be doubled and this process called as DNA amplification (Dennis et al., 2006).

DNA sequencing is a method that gives precise knowledge about the order of the nucleotides in a DNA molecule. It is crucial to know sequence of a barcode gene to make species identification. There are a lot of sequencing methods like Maxam-Gilbert sequencing, Chain-termination sequencing, massively parallel signature sequencing, polony sequencing, pyrosequencing, Ion Torrent semiconductor sequencing, etc. In this research, the Chain-termination sequencing method was used (Akeson et al., 2001). Chain-terminated sequencing is based on the classic polymerase chain reaction, but it includes modified nucleic acids to terminate the reaction. These molecules known as

dideoxynucleoside triphosphates (ddNTP) and depend on the sequencing technology they can be tagged by different molecules like fluorescence or radioactive molecules. Then, the terminated sequences are aligned with electrophoresis and sequence data would be obtained (Work et al., 1983).

In order to get useful results from the raw sequence data, some Bioinformatics tools should be used. First of all, the sequence chromatograms must be cleaned up according to their quality score. Then, the clean sequence data should checked with a BLAST tool on the NCBI nucleotide database to find a match with a specific species. If there is a match higher than 97% similarity, it is convenient to proclaim the organism's species. Otherwise, depend on the similarity organism could be identified as gender, family or order level.

In some cases, a taxonomic tree can be helpful to determine the taxonomic group of the unknown organism. In order to calculate a taxonomic tree, all of the sequences must be aligned by multiple sequence aligner software. After, they are converted to a suitable file format according to the consistency between alignment and tree calculating software. Then, the taxonomic tree is calculated by software. However, the tree algorithms must be chosen carefully according to aim of the research. Finally, taxonomic tree must be investigated according to bootstrap scores of the branches and then the taxonomic relationship can be interpreted (Baxevanis et al., 2001).

1.1.3. HRM Analysis in Species Identification

High resolution melting (HRM) is a technique that characterizes the short amplicon fragments according to their sequences. This method detects up to one base difference, hence it can be used for mutation detections, sequence matching and multiplex genotyping analysis. HRM analysis uses the raw melting curve data from the qPCR output. Then the data set of the melting profile is normalized and the normalized data is used to construct a difference plot which makes possible to detect small sequence variations among the data set (Ajitkumar et al., 2013).

QPCR is a molecular biology technique which multiplies the number of the target DNA amplicon as classic PCR while it detects the synthesized amplicons in real-time. The reaction is not different than the conventional PCR but the distinctive point is the detection part. The detection can mainly be in two ways, whether by using double stranded DNA binding fluorochromes or using the hybridization probes as reporters (Mackay, 2007). These two reporter systems have both advantages and disadvantages depend on the experiment, but in order to accomplish the HRM analysis, double stranded DNA binding dyes must be used. These dyes are intercalated into the DNA when it is in double stranded formation. In this formation these dyes have significant light emission which is used to calculate double stranded DNA amount (Dorak, 2007). When the temperature increases, the heat energy breaks the hydrogen bonds of the DNA molecules and the DNA molecules become single stranded. In this formation of DNA, intercalating dyes separate from the DNA and they do not give any significant emission. Therefore, at the end of each polymerase chain reaction cycle, a light source sends a beam in a specific spectrum to excite the dyes and a detector reads the emission which is proportional to the amount of dsDNA currently present (Mackay, 2007).

After the qPCR is done, melting curve analysis is carried out to examine the characteristics of the multiplied sequence. Basically, melting curve is a graph where the x-axis demonstrates the temperature and the y-axis shows the fluorescence amount. When the temperature slowly increased, the hydrogen bonds of the DNA begin the separate. Consequently, the emission strength of the molecules begins to diminish as the temperature increases. This diminishes in the amount of fluorescent emission continue until all the DNA molecules are degraded into single stranded. When the half of the DNA is denatured, this point called as melting temperature (Tm) which is usually distinctive characteristics of the amplicons depend on the sequence length and base content (Mackay, 2007). A melting curve profile and melting temperature is illustrated in the figure 1.4.

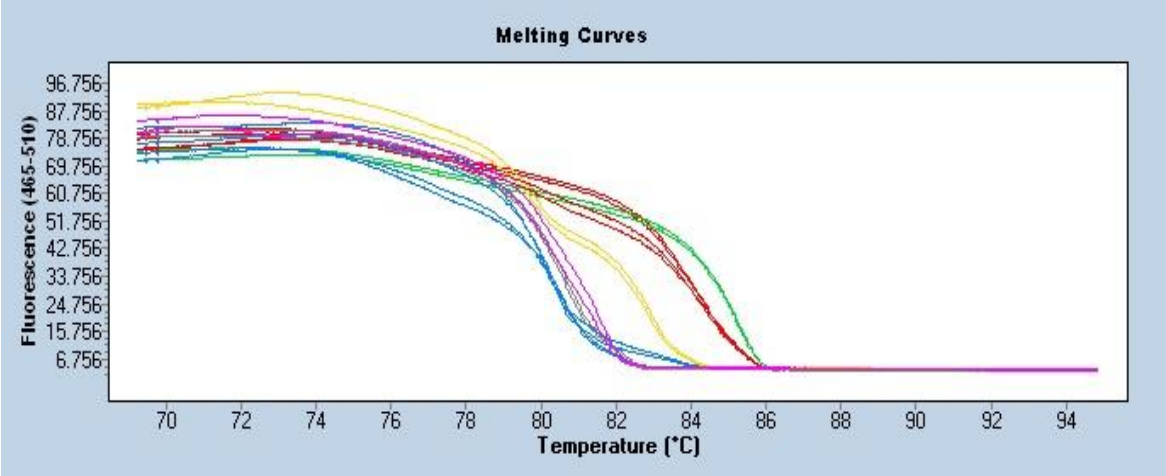

Figure 1.4. A melting curve data acquired from Roche LightCycler 480 device (unpublished data).

In order to draw a HRM curve, the raw data of melting profile must be normalized. The raw data composed from temperature versus fluorescent amount. The normalization step converts the fluorescent amount into 0% to 100% scale. (Reja et al., 2010) Therefore, after normalization, x-axis stayed as temperature, but the y-axis changed into fluorescent rate as indicated in the figure 1.5.

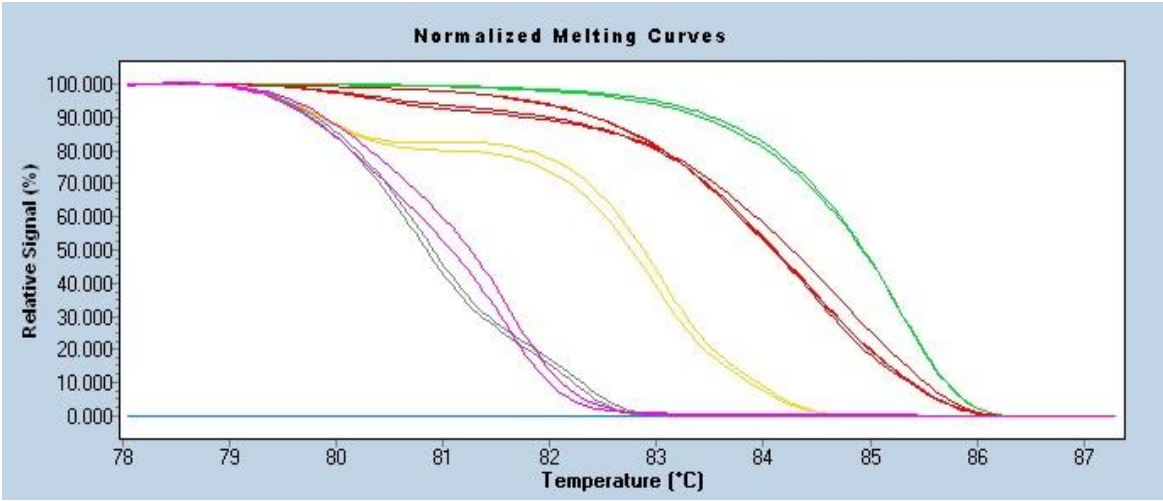

Figure 1.5. Normalized melting curve data acquired from Roche LightCycler 480 device (unpublished data).

Difference plot is used to cluster the melting curve profiles automatically. A reference curve is subtracted from all other curves (Reja et al., 2010), and the result become clearer as demonstrated in the figure 1.6.

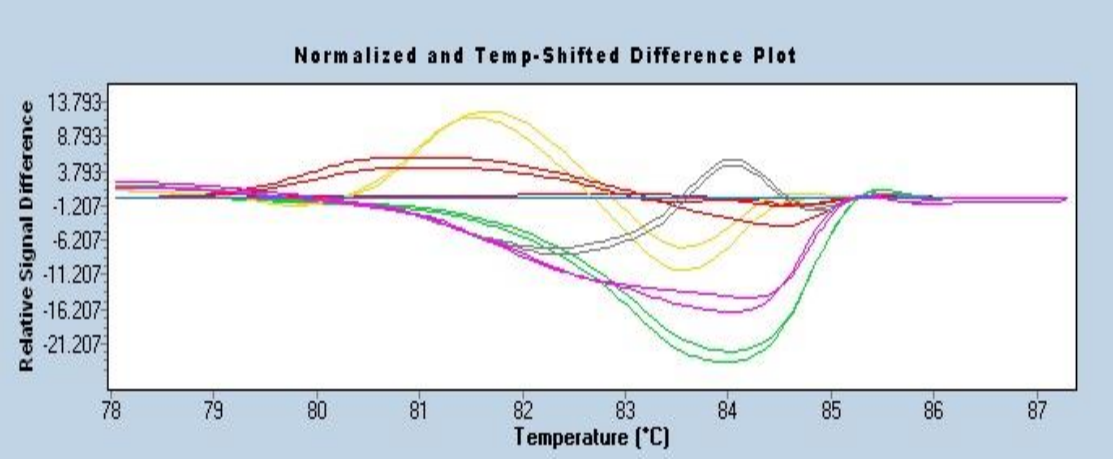

Figure 1.6 Difference plot data acquired from Roche LightCycler 480 device (unpublished data).

2. STATEMENT OF PROBLEM

Zooplankton diversity is an important parameter to monitor marine ecosystem. However, identification of Zooplankton species is not an easy task due to their broad diversity. Morphological identification or DNA Barcoding are not suitable methods for routine monitoring studies. Therefore, there is a need for a new methodology for identification of Zooplankton species. On the other hand, the Black Sea is one of the largest marine ecosystems in the world, yet the Zooplankton diversity is not entirely known.

Therefore, this study aims that to develop a new method for rapid determination of Zooplankton species in the Black Sea. This method gives the results in approximately 3 hours. The simplicity and easiness of the technique enable its use on both research stations and ships, thus rendering it likely to become a routinely employed method for identification studies. The outcomes of this project could be used both in routine biodiversity investigations and in ecosystem management in Turkish coastal waters where the impacts of invasive species are strong. Also, the investigation about the Zooplankton diversity of the Black Sea is one of the other main outcomes.

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3. MATERIALS AND METHODS

3.1. Sampling

Sampling was conducted by İlayda Destan Öztürk with R/V Bilim2 ship (Institute of Marine Sciences, Middle East Technical University, Turkey) from 7 stations throughout the Turkish coast of the Black Sea in the scope of DEKOSIM and Perseus projects. These stations were located on Şile, Sinop, Boğaz and Trabzon as indicated in figure 3.1. Besides, the coordinates of the sampling areas are demonstrated in the table 3.1. The samples were collected from benthic layer and the surface layer of the sea. Sampling was performed one time for each station during the summer season in 2013.

Figure 3.1. location of the sampling stations in Turkish coast of Black Sea.

Table 3.1. Coordinates of the sampling stations

Station number	Region	Longitude	Latitude	Date	Depth	Type
1	Boğaz	29,1333	41.225	13.6.2013	50	Vertical
2	Şile	29.604	41.237	5.8.2013	0	Horizontal
3	Şile	29.647	41.343	5.8.2013	0	Horizontal
4	Sinop	35.204	41.951	23.7.2013	133	Vertical
5	Sinop	35,45	42,3	23.7.2013	65	Vertical
6	Trabzon	39,75	41,25	28.7.2013	163	Vertical
7	Trabzon	39.725	41,06	30.7.2013	175	Vertical

In order to collect Macrozooplankton, three methods were used. Firstly, WP2 and Hensen nets were used to collect Macrozooplankton species from ship. The sampling was carried out vertically from the sea floor in the shallow areas and from the beginning of the Hydrogen Sulphide layer in the deep areas of the Black Sea. The samples were grouped and labeled in the laboratory of the ship.

Secondly, some common benthic species like mussels, sponges and gastropods were collected by snorkeling from the coastal regions of Boğaz, Şile, Sinop and Trabzon in the scope of COCNET project.

Finally, some fish species were obtained from the local fisherman whether they have economic value or not. These species were turbot, anchovy, horse mackerel, perch, haddock, goby and scorpion fish. After each labeled samples were taken photograph, depends on the organism, 1-2 cm^3 of tissue or the whole organism was stored for the molecular analysis. The samples were preserved in 70% ethanol solution and at -20 ^{0}C temperature until the DNA analysis. After that the unspoiled samples were stored as

museum samples. The labeling studies carried out in the laboratories of Institute of Marine Sciences of Middle East Technical Universitiy.

For Mesozooplankton sampling, the same WP2 and Hensen nets were used to collect them as Macrozooplankton sampling. After Macrozooplankton samples were picked from the nets, the Mesozooplankton samples were taken and preserved in % 70 ethanol solutions. Photographs of the Mesozooplankton samples were taken under the microscope and each sample placed in a separate Eppendorf tube in order to ease DNA extraction by İlayda Destan Öztürk and Dr. Yeşim Ak Örek.

3.2. Molecular Design

In order to amplify the COI gene of the Zooplankton and fish species, primers were designed or taken from the related articles. In case of failure in amplification step, many different primer pairs were prepared. These were mainly, degenerate primers, species specific primers and the primers chosen from relative studies.

The potentially expected Zooplankton species in the Black Sea are listed in the table 3.2 (Vershinin, 2007). The sequences for these species were obtained from NCBI and BOLD databases. Some of the species have not COI gene sequences in the databases. These species were excluded from the list. The COI gene sequences of these organisms are given in the appendix A.

Table 3.2. Potentially expected Zooplankton species in the Black Sea

Zooplankton Species	
Oithona similis	*Phyllodoce sp.* *(groenlandica)*
Paracalanus parvus	*Mytilus galloprovincialis*
Pseudocalanus elongatus	*Scapharca inaequivalvis*
Acartia tonsa	*Hydrobia acuta*
Acartia clausii	*Retusa sp*
Calanus euxinus	*Haminoea navicula*
Penilia avirostris	*Tergipes tergipes*
Evadne spinifera	*Upogebia*
Evadne nordmanni	*Xantho poressa*
Pleopis polyphemoides	*Rhithropanopeus harrisii*
Pleurobrachia pileus	*Crangon crangon*
Mnemiopsis leidyi	*Palaemon elegans*
Aurelia aurita	*Athanas nitescens*
Engraulis encrasicolus (Larvae)	*Alpheus sp.*
Trachurus mediterraneus	*diogenes pugilator*
Mullus barbatus	*Pachygrapsus marmoratus*
Diplodus annularis	*Macropodia sp.*
Scolelepis squamata	*Balanus improvises*
Alitta succinea	*Phoronis sp.*
Nephtys hombergii	*Verruca sp.*
Pholoe sp.	*Botryllus schlosseri*
Sagitta setosa	*Noctiluca scintillans*

Sequences in the appendix A. were edited into fasta format and uploaded to Clustal X software (Larkin et al., 2007). Sequences were aligned by this software according to their sequence similarities and the result was saved in nexus format.

Aligned sequences were uploaded to MEGA 6 software and the data in the nexus format converted into mega format. Then, the sequences were used to calculate a tree in neighbor joining method. The parameters in the program are indicated in the Appendix B. After the tree was calculated, the main branches were separated into six groups as presented in the figure 3.2.

These steps were repeated for different phylogeny methods. Parameters and the trees are illustrated in Appendix B for maximum likelihood method, UPGMA method and minimum evolution method.

According to figure 3.2, sequences in the six branches of the tree were separately aligned by using PrimaClade software. The alignments were used to create degenerate primers. Also all of the sequences were aligned together to make a degenerate primer which might be anneal all of the targets. The primer regions were selected heterogeneously hence; the targeted sequences of COI gene were exhibited variation in length and position. The degenerate primers are presented in table 3.3.

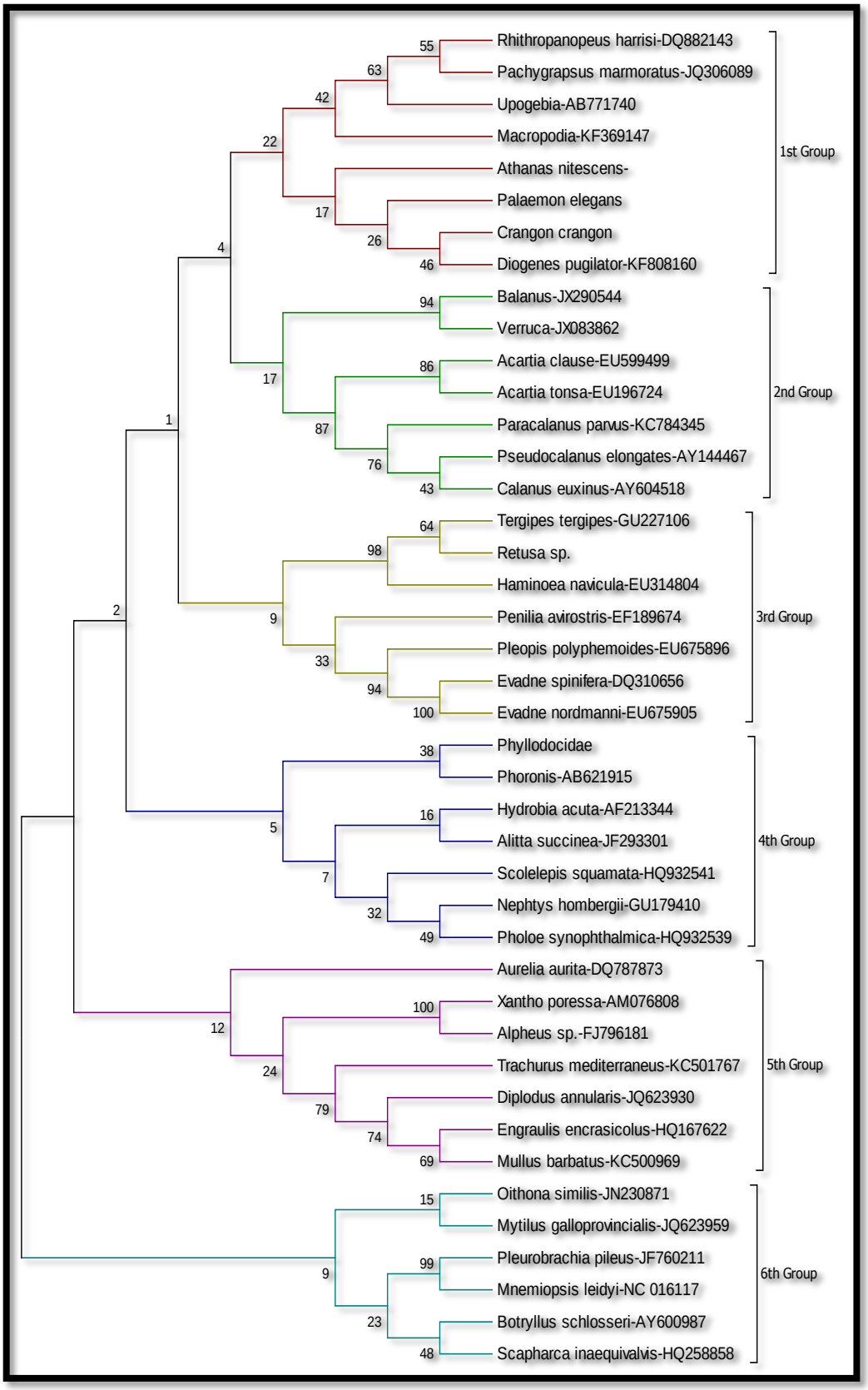

Figure 3.2. MtCOI targeted similarity tree constructed with Neighbor joining method

Table 3.3. Sequences of Degenerate Primers for COI gene of the taxonomically organized Zooplankton groups

Primer Name	Primer's Target Groups	Sequences of Forward Primer (5'-3')	Sequences of Reverse Primer (5'-3')
deg-1	group 1	ATTGGNGGVTTYGGDAAYTG	ATAAARTTWAYDGCHCCTARRAT
deg-2	group 2	ATRGTWATRCCDATYHTAATTGG	RCHCCNGCWARRTGHARNGA
deg-3	group 3	ACVGCYCAYGGYBTTYRTWAT	CGTATRTTHAHRATHGTMGTAAT
deg-4	group 4	TDGGWRRDGAYCARYTDTAYAA	GARAAAATDGCWARRTCWAC
deg-5	group 5	CHRTYDKDRTHSKRGGVTT	AAGAAVGHDGWRTTKARRTTT
deg-6	group 6	ATHRKDDSKTYWKBWAATT	HHBHDHNKBDBHRDWAAA
deg-all	All groups	BDRTHVKNDSNTYHKBNRWHT	GAYWRKNDWNRYHHMDDYVNRH

Due to lack of quality of designed degenerate primers, species specific primers were also designed by using NCBI Primer Blast tool. COI sequences of the species were used as the template and the length of the target amplicon was designed to be between 300bp and 500bp. After the Primer Blast calculated the candidate primer sequences the best candidates were selected according to amplicon length, mispriming, mismatch, specificity and various other parameters. The designed primers are indicated in the Appendix C.

In order to amplify the all of the species with one or two primer pairs, the universal primers were selected from previous studies as presented in table 3.4.

Table 3.4. Selected primers from previous studies for mt COI gene amplification

Primer Name	Target Organisms	Primer Sequences (5'-3')
LCO1490	Metazoa	GGTCAACAAATCATAAAGATATTGG
HCO2198	Metazoa	TAAACTTCAGGGTGACCAAAAAATCA
L5950	Universal	ACAATCACAAAGAYATYGG
H7196	Universal	AGAAAATGTTGWGGGAARAA
dgLCO1490	Metazoa	GGTCAACAAATCATAAAGAYATYGG
dgHCO2198	Metazoa	TAAACTTCAGGGTGACCAAARAAYCA
uni-minibarF	Metazoa	TCCACTAATCACAARGATATTGGTAC
uni-minibarR	Metazoa	GAAAATCATAATGAAGGCATGAGC
mlCOIintF	Universal	GGWACWGGWTGAACWGTWTAYCCYCC
mlCOIintR	Universal	GGRGGRTASACSGTTCASCCSGTSCC
Fish-BCL	Fish	TCAACYAATCAYAAAGATATYGGCAC
Fish-BCH	Fish	ACTTCYGGGTGRCCRAARAATCA
COI VF1	Fish	TTCTCAACCAACCACAAAGACATTGG
COI VR1	Fish	TAGACTTCTGGGTGGCCAAAGAATCA
jgLCO1490	Metazoa	TITCIACIAAYCAYAARGAYATTGG
jgHCO2198	Metazoa	TAIACYTCIGGRTGICCRAARAAYCA

LCOI1490/HCO2198 primer pairs were targeted the 710 bp part (Folmer et al., 1994), L5950/H7196 primer pairs were targeted 1264 bp part (Avise, 1994), dgLCO1490/ dgHCO2198 primer pairs were targeted 710 bp part (Meyer, 2003), Uni-minibarF/Uni-minibarR primer pairs were targeted 130 bp part (Meusnier et al., 2008), mlCOIintF/ mlCOIintR primer pairs were targeted 313 bp part (Leray et al., 2013), Fish-BCL/ Fish-BCH primer pairs were targeted 648 bp part (Baldwin et al., 2008), COI VF1/ COI VR1 primer pairs were targeted 710 bp part (Gleason et al., 2012), jgLCO1490/ jgHCO2198 primer pairs were targeted 658 bp part of mtCOI gene in most of the animal species.

3.3. DNA Extraction

The organism groups were included fish and Zooplankton species. Hence, their size and physiology shows a great variation. In order to extract the mitochondrial DNA of these species Chelex 100 method was used.

DNA extraction method with Chelex basically consist of three steps; homogenization of tissue, cell disruption and DNA separation. At each step, different parameters were introduced to see any change in the DNA extraction quality for both fish and Zooplankton species. Firstly, tissue amount was assessed by changing the initial sample mass. Then, the tissue parts of the species were examined, but only for fishes. Afterwards, homogenization step with different size of glass beads were checked up. Moreover, mixing speed, mixing time, incubation temperature, incubation time, chelex percentage in solution and total solution volume were optimized with parallel experiments. DNA extraction procedures were formed as a result of these optimization steps.

$2mm^3$ of tissue is taken from the caudal part of fish, placed into an Eppendorf tube. Then, 300 µl, %10 chelex solution is added in to the Eppendorf tube. The mixture is homogenized with vortex for 1 minute. Quick spin is performed. Afterwards, the mixture is placed on a heat block and incubated at 98 ^{0}C for 10 minutes. After incubation, the mixture is homogenized at 98 ^{0}C for 5 minutes at 3000 rpm. The mixture is centrifuged for 5 minutes in room temperature at 14500 rpm. 100 µl of supernatant is gently removed from the mixture and saved in a new Eppendorf tube. Finally, the remaining of the mixture is discharged

Zooplankton sample as a whole, placed into an Eppendorf tube. Then, 200 µl, %10 chelex solution is added in to the Eppendorf tube. The mixture is homogenized with vortex for 1 minute. Then, quick spin is performed. The mixture is placed on a heat block and incubated at 98 ^{0}C for 10 minutes. After the incubation, lids of the Eppendorf tubes are checked to avoid any leaks of liquid. The mixture is homogenized at 98 ^{0}C for 5 minutes at

3000 rpm. The mixture is centrifuged for 5 minutes at room temperature at 14500 rpm. 100 µl of supernatant is gently removed from the mixture and saved in a new Eppendorf tube. When there are not enough chelex-free layers in the supernatant, then less than 100 µl samples are obtained. The remaining of the mixture is discharged.

Blank was performed by molecular biology grade water (used as a solvent), 2 µl of sample (including blank) were placed on the tip of spectrophotometer (MSP-100, inovia technology). In order to measure, spectrophotometer software was configured for dsDNA and a UV length of 260 nm. The measurements were performed according to the manufacturer's manual. Concentrations and UV 260/280 ratios were measured and recorded

3.4. Quantitative Polymerase Chain Reaction

Reaction ingredients were prepared as indicated in the table 3.5. Then the reaction solution was placed in 96-well plate and inserted into a Roche LightCycler 480 device.

Table 3.5. reaction ingredients and their optimized amounts

Reactants	**Amounts**
Reaction mix	5 µl
Forward Primer	0,5 µl
Reverse Primer	0,5 µl
MG Water	3 µl
Template	1 µl
Total	10 µl

In order to multiply the COI genes, suitable programs were written. These programs were optimized for the primer pair and DNA template. QPCR programs for fish and Zooplankton species are indicated in table 3.6 and table 3.7.

Table 3.6. QPCR program for amplification of fish species

Step	**Time**	**Temperature**	**Cycle**
Initial Denaturation	5 min	98^0C	1
Denaturation	15 s	98^0C	45
Annealing	15s	65^0C	
Extension	30s	72^0C	
HRM	5 sec/step	72^0C -95^0C ($0,2^0$C inc.)	1
Cooling	-	37^0C	1

Table 3.7. QPCR program for amplification of Zooplankton species

Steps	**Time**	**Temperature**	**Cycle**
Initial Denaturation	3 min	98^0C	1
Denaturation	10 s	98^0C	55
Annealing	10s	52^0C	
Extension	15s	72^0C	
HRM	5 sec/step	72^0C -95^0C ($0,2\ ^0$C inc.)	1
Cooling	-	37^0C	1

In order to amplify fish species, COI VF1 / COI VR1 primer pairs were used. Similarly, to amplify Zooplankton species, LCOI1490 / HCO2198 primer pairs were used.

3.5. DNA Sequencing

The amplicons were purified and sequenced using the ABI prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit on an ABI Prism 377 DNA sequencer (Applied Biosystems, USA).

After the sequence results were obtained, sequences visualized by DNA baser software. Then, the sequences were cleaned until they became reliable results. In order to clean sequences, both ends were trimmed until they have a 50 % average quality or above. Afterwards, the trimmed sequences were uploaded into the NCBI database to find related matches. In order to molecularly identification, nucleotide Basic Local Alignment Search Tool (BLAST) of NCBI database was used. Moreover, megablast, discontiguous blast and blastn algorithms were used together to find out the most accurate results for the sequences which were not matched with an organism in high similarity percentage. Finally, these sequences were used to calculate phylogeny trees.

Phylogeny trees were calculated by MEGA 6 software as indicated in the degenerate primer design. These trees were used to estimate species of the organisms or at least their closest taxon.

3.6. HRM Software

The application was written in C# language using .NET framework which made the software cross-platform. The software was designed to keep data in a database which allows the user to insert new data sets. The application was developed to read various input formats including MS Excel worksheets.

Firstly, the data was read from input file which has been taken from Roche LightCycler 480 device. Then, the data were plotted on a 2d coordinate system. Next, pre-melt and post-melt areas were selected respectively.

A reference data set was selected as a base sample. A line of best fit for the pre-melt and post-melt areas was computed for this sample. All of the other samples were forced to have the same line of best fit for pre-melt and post-melt areas by rotating and shifting them accordingly. The plot was converted to 0-100 scale as relative signal, instead of real fluorescence values. Additionally, new curves were plotted from the beginning of the pre-melt zone until the end of the post-melt zone.

All of the normalized data sets were sequentially subtracted from the base sample and the difference was plotted. The subtraction process was carried out point by point through the all relative signal values against the temperature.

Data grouping was made by computing the Euclid Distance between the Difference curves. An accuracy threshold was chosen by the user according to data quality. Two samples were grouped if the Euclid Distance between the fluorescent values were smaller than the accuracy threshold for all temperature values.

A database was built by inserting the whole base samples, the pre-melting and post-melting zone coordinates and all the difference plot data. Then, the organism names were placed in related difference plot lines. Therefore, the software was determined the species automatically from the melt curve data.

Since the species identification or grouping is managed by computing the distance of difference curves, a tool is added to software for controlling the error margin. Thus, users manually adjust the best error limit for its own experiment.

4. RESULTS AND DISCUSSION

4.1. Taxonomic Findings

Collected samples were investigated under the microscope and all fish species which were identified given in table 4.1, also the identified Zooplankton species given in table 4.2.

Table 4.1. Taxonomically identified fish species in the Black Sea

Species name	**Location**
Sarda sarda	Şile
Spicara flexuosa	Şile
Scorpaena sp.	Şile
Helicolenus dactylopterus	Şile
Engraulis encrasicolus	Şile
Merlangius merlangus euxinus	Şile
Alosa pontica	Şile
Mullus barbatus	Şile
Gobius niger	Şile
Gaidropsarus mediterraneus	Şile
Chelidonichthys lucerna	Şile
Psetta maxima	Şile
Serranus hepatus	Şile
Squalus sp.	Şile
Raja sp.	Şile
Platichthys flesus	Şile
Trachinus draco	Şile
Neogobius melanostomus	Şile

Gobius niger	Şile
Asterias rubens	Şile

Table 4.2. Morphologically identified Zooplankton groups and species in the Black Sea

Zooplankton Species	
Oikopleura dioica	*Paracalanus parvus*
Bivalvia	*Oithona similis*
Brachyura	*Ctenophora (cydipid)*
Brachyura Megalopa Larvae	*Decapod*
Bryozoa	*Decapod Larvae*
Bryozoa Cyphonautes Larvae	*Engraulis encrasicolus egg and larvae*
Chaetognatha	*Pseudocalanus elongatus*
Sagitta setosa	*Gastropoda*
Cephalochordata	*Hydromedusae*
Cirripedia	*Isopoda*
Cirripedia Larvae	*Jellyfish ephyra*
Cladocera	*Noctiluca scintillans*
Evadne tergestina	*Ostracoda*
Penilia avirostris	*Polychaeta*
Podon sp.	*Teleostei Larvae*
Evadne spinifera	*Aurelia aurita*
Copepoda	*Beroe ovata*
Calanus euxinus	*Mnemiopsis leidyi*
Acartia sp.	*Pleurobrachia pileus*
Centropages ponticus	*Rhizostoma pulmo*

In addition, all of the Zooplankton samples were photographed under the microscope. The images of the selected organisms can be seen in the Appendix D.

Expected species in the Black Sea are given in the table 3.2. and the taxonomic findings are given in the Appendix F. The findings are consisted with the expectations in general. However, there are some species which are not found and some they are not expected. The species that not found could be missing from sampling zone. Other than that seasonal temperature fluctuation could cause a drop in the population because the sampling was performed one time for each station (Ak et al., 2012).

Some unexpected Zooplankton could be misidentified by taxonomists. It is quite possible for some of the unexpected Zooplankton species when the results are compared with the sequence based identification (Katz et al., 2009). Another reason could be the migrating species. The Bosporus is a bridge between the Black Sea and Marmara Sea. Thus, it is very possible to find some species from Marmara Sea or even Mediterranean Sea (Kovalev et al., 1999).

4.2. Optimization Results

Various optimizations were done for DNA extraction and qPCR experiments to get better results. As mentioned in the methods, a series of experiments were carried out for optimization of the DNA extraction according to previous studies (Yue et al., 2001), (Aranishi et al., 2006), (Simonelli et al., 2009), (Fanggang et al., 2008). Optimization of DNA extraction was differed for fish and Zooplankton organisms. For fish species, different amount of starting materials was tested and 2 mm^3 of the sample was found as optimal. Then, homogenization step was tested with 0.5 mm and 0.1 mm glass beads, but there was not any improvement, ergo bead step was discarded. The extraction solution was tested for 5%, 10% and 20% chelex concentrations and 10% chelex concentration was found as the best amount. Additionally, mixing times for each step, solution volume, centrifugation speed and time were tested and the optimal protocol shaped as mentioned in

the methods. After the fish species, almost same optimization steps were repeated for Zooplankton species. However, the Zooplankton species were used as whole organisms because their body mass relatively much smaller than fishes. The protocol for Zooplankton species is shaped as mentioned in the methods.

Optimization of DNA extraction was performed in aspects of tissue amount, homogenization, chelex concentration, total solution volume, temperature, centrifugation and step times (Kjärstin et al., 2004). When different amounts of starting materials were tested, it seemed that the DNA isolates from high amount of tissue inhibited the PCR reaction. On the other hand, when the tissue amount was lowered too much than the Ct values were too high. Therefore, approximately 2 mm^3 of tissue was provided best result. Homogenization step was checked with temperature, vortex and beads. Apparently, beads had no effect on the extraction quality, but the heat and vortex were enough for homogenization step. The temperature was adjusted as high as it gets without reaching the boiling point. Centrifugation was performed at highest speed of currently possessed machine which was 14500 rpm. Centrifugation period was tested in different times and it was concluded that 5 minutes was optimal duration. After 5 minutes of centrifugation DNA was precipitated and the yield was too low, but before the 5 minutes the PCR inhibitors were not eliminated properly.

QPCR optimization was done by adjusting the primer concentration, sample dilution, sample volume, temperature and time of amplification cycle steps (Raymaekers et al., 2009). Thus, a series of primer concentrations were tested for all universal primers and the best result was taken from 0.5 µl of each LCOI1490 / HCO2198 primer pairs for Zooplankton species (Folmer et al, 1994) and 0.5 µl of Fish-BCL / Fish-BCH primer pairs for fish species (Baldwin et al., 2008), while the total reaction volume was 10 µl for each well. DNA templates were diluted in 10 fold, 100 fold and 1000 fold to find out the most efficient amount. Best result was 1µl of 100 fold diluted template for 10 µl total reaction volume. Simultaneously, amplification steps were optimized by changing temperature and time intervals of denaturation, annealing and extension steps. According to fluorescent

signal strength, cycle number was determined as demonstrated in table 3.6 and 3.7 for fish and Zooplankton species respectively.

After the DNA extraction and qPCR optimizations were handled, the primer pairs were tested to find out the best results. The aim was to amplify all samples with one primer pair. Consequently, best result was obtained by using LCOI1490 / HCO2198 primer pairs for Zooplankton species and Fish-BCL / Fish-BCH primer pairs for fish species. However, some organisms were amplified better with different primer pairs. This situation led us to use mixed primer cocktails. Even though, the primer cocktails were checked about their compatibility about annealing temperature and hetero-dimer complexes, the outcome was a failure. Therefore, it was decided to use best candidates for further steps.

Next step was to find out their optimal annealing temperatures. For this reason, a series of experiments with different annealing temperatures was carried out since the Roche LightCycler 480 machine have not gradient feature. The reaction efficiency was improved with increasing the primer concentration up to 0.5 µl. Then, denaturation, annealing and extension periods were optimized with a series of experiments.

4.3. Amplification and Melting Curve Profiles

QPCR experiments were performed by using Roche LightCyler 480 device. Amplification and melt curve graphs are presented in Appendix E. All samples were duplicated, ergo using these plots the better curves were chosen for sequencing and HRM database. Samples were selected according to fluorescent level, and smoothness of the curves. Generally, amplification curves were exceeded the Ct value after 35 cycle for Zooplankton samples. However, Ct value is nearly 10 cycles earlier for fish species than the Zooplankton. Melting peaks were between 78 ^{0}C and 86 ^{0}C in general but there were some Zooplankton samples that they had multiple peaks consistently.

QPCR results were obtained as amplification and melting curves. Amplification curves were not in ideal shape for Zooplankton species but that was insignificant since the HRM analysis was needed only the melting curve data. Melt peaks were between 78 ^{0}C and 86 ^{0}C for most of the samples. Some of them had multiple peaks which could be a problem, if they were not gave the same pattern with the duplicates and the parallel samples. Therefore, this abnormal patterns were actually contributed the discrimination of the different samples. At first, this anomaly considered as a contamination or experimental failure. However, the consistent results show that they were characteristic to the sample type.

QPCR results were organized and filtered according to amplification and melting curve success. Then the qualified samples are separated to sequencing and HRM database construction. General profiles of amplification and melt curves were given in Appendix E. This results were comprised all of the raw data without filtering. Considering the filtering is a long process, the cleaned data is not given one by one. However, all the HRM profiles including the melting curve were calculated by using the filtered data.

Difference plots were calculated by Gene Scanning tool of Roche LightCycler 480 device. Difference plot graphs are demonstrated in Appendix E. Gene Scanning software were separated all of the samples in 8 groups which is the limit for this software. As shown in the figures, difference plot curves are more different from each other than the melt curves. For difference plots, base sample was chosen as human DNA sample which was used as positive control during entire qPCR experiments.

4.4. Sequence Results

Sequence results were taken in “ab1” format and uploaded into DNA Baser software v2.71.0 (Heracle Software, Lilienthal, Germany). This software cleaned the sequences according to their quality scores. Thus, the cleaned sequences were uploaded Blast tool on the NCBI database to find out their species (Bucklin et al., 2007).

Morphological and molecular based findings were given in the Appendix F. In general, morphological identification is more valid than the molecular identification. Thus, when there was confusion about the identification of the sample, morphological identification accepted as the reference method (Keskin et al., 2013). In many cases, the morphological identifications had less taxonomic resolution than the molecular methods. In those circumstances, sequence based results were checked whether they parallel with the taxonomic findings.

The findings in the Blast were not easily led the conclusion all the time. Some parts of the sequences were matched with high similarity, but they were not belonged the Zooplankton species. These samples were obviously contaminated samples. Some of them were matched with low quality even though they were matched with the taxonomic expectations, they were still eliminated. Other than these, some of the samples were matched with respectively low quality, but their taxonomic candidates were not in the database. Therefore, it is a high possibility that it was the first time these COI sequences were sequenced. However, these organisms were also classified into two groups, one of them was highly similar with the closest taxon and the other one was lowly similar. The first of them were separated as the new sequence sources for the database yet the second situation was remained to be further investigated.

Phylogenetic tree was calculated for better understanding the kinship of organisms. As mentioned above, some of the organisms were hard to identify. In these situations, the phylogenetic tree became very helpful to visualize their taxonomic positions. Since all of the sequences obtained from COI gene, it was ideal to construct a phylogenetic tree. The phylogenetic tree constructed by MEGA 6 software. Even though it is the slowest method, maximum likelihood method is chosen because it is more confident than the other methods, it draws all possible trees and chooses the most suitable ones (Reddy, 2011).

In the previous studies, mtCOI is used as a successful phylogenetic marker for, ciliated protists (Zhao et al., 2013), Culicoides imicola species (Linto et al., 2002) and

Calliphorinae fly species (Park et al., 2009). However, in this study, quality of phylogenetic tree is relatively low according to bootstrap values as indicated in figure 4.3.

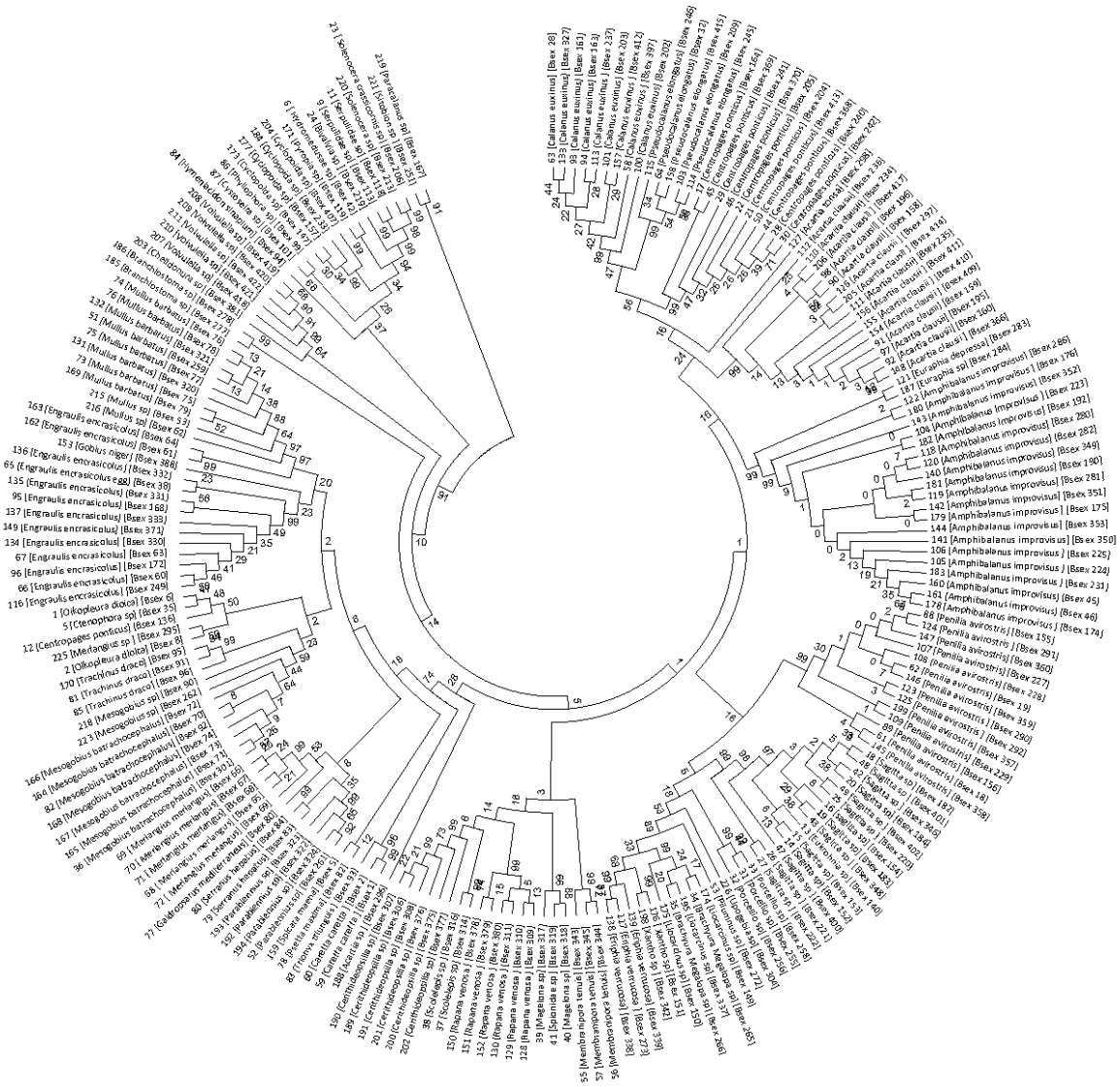

Figure 4.1. Phylogenetic tree constructed with maximum-likelihood method for all sequences

Nevertheless, phylogenetic tree was presented a valuable clue for the determination of the species, whether sequence based results or morphological results were more suitable. Especially, when there was confusion about the results, the tree could be very useful.

When sequences were uploaded into the Mega software, protein-coding data box was checked since the COI is a protein-coding gene (Folmer et al., 1994). Then, phylogenetic tree was calculated by the maximum-likelihood algorithm. This algorithm calculates all possible trees and draws the most possible consensus tree. Therefore, it takes time, but the results are considerably more accurate than the others (Tamura et al., 2011). The results of phylogenetic tree are very parallel with the findings from NCBI database. This outcome is of course not a surprise considering the maximum-likelihood method used the sequence data for calculation of the phylogenetic tree.

As shown in the figure 4.1, the tree already compatible with the expectations. However, for some organisms, bootstrap values very lower than expected. The main reason of this result is Zooplankton covers a great diversity of organisms (Varadharajan et al., 2013). Therefore, organisms are genetically distant from each other. As a matter of fact, this situation demonstrates a very important advantage that COI indeed a valid marker for Zooplankton species. Thus, COI sequences show low similarity among the species and higher similarity within the species for Black Sea Zooplankton.

4.5. HRM Software Results

HRM software was operated by using the raw qPCR melt curve datasets. These datasets composed of the list of relative fluorescent unit values for each temperature value. The increment of the temperature was 0,02 ^{0}C per second and acquisition rate was 25 per 1 ^{0}C. Firstly, the melt curves were plotted by HRM software. Secondly, melt curves were normalized to 0-100 scale for all samples. Then, normalized curves were transformed into difference plots as indicated in Appendix G.

The database was created from representative difference plots of some selected organisms. Since there were more than one melting curve data for most of the organisms, the matching performance of the software was relatively tested by using the other samples. Species which are used for creating database are shown in the Table 4.1.

Table.4.3. Species list of the HRM database

Species
Merlangius merlangus
Acartia clausii
Amphibalanus improvisus
Calanus euxinus
Engraulis encrasicolus
Mesogobius batrachocephalus
Mullus barbatus
Penilia avirostris
Pseudocalanus elongatus
Rapana venosa

After the database was created, the software was used to analyze all of the data as indicated in figure 4.2, 4.3 and 4.4. The findings were too complicated to comment for such a big data group. Therefore, data sets were uploaded to the software with smaller groups. In addition, to validate matching mechanism of the software, these groups were formed from samples which were considered as the same species. The results of these groups are demonstrated in the Appendix G.

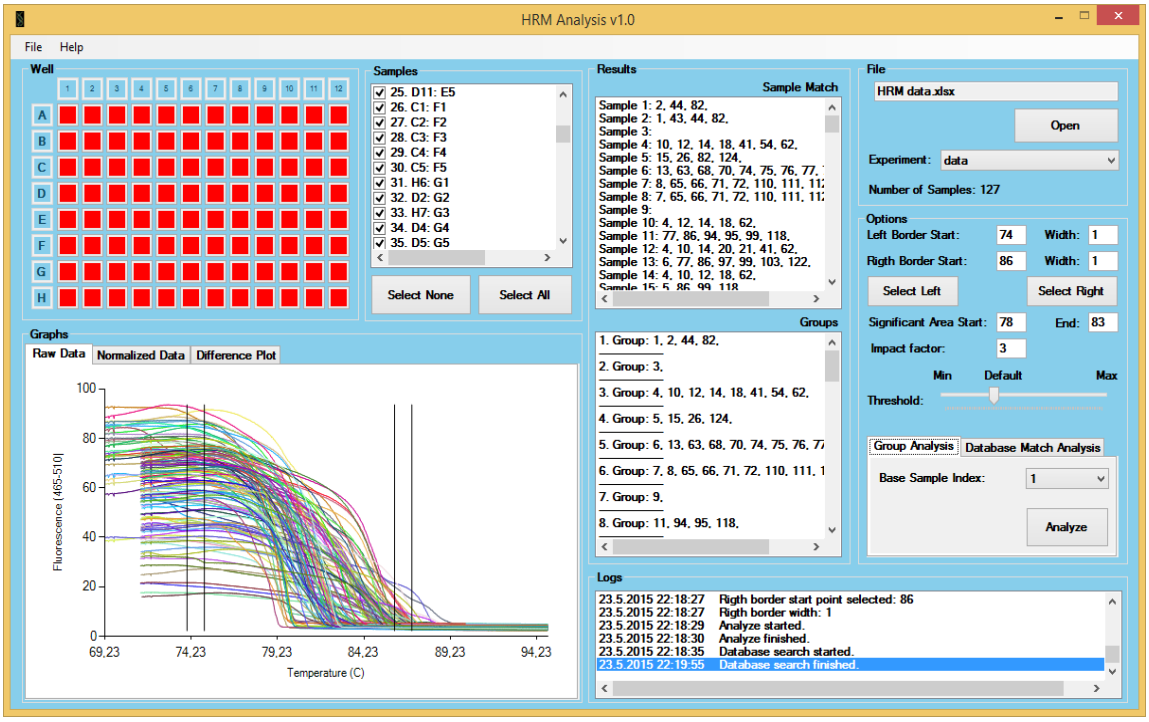

Figure 4.2. Phylogenetic tree constructed with maximum-likelihood method for all sequences

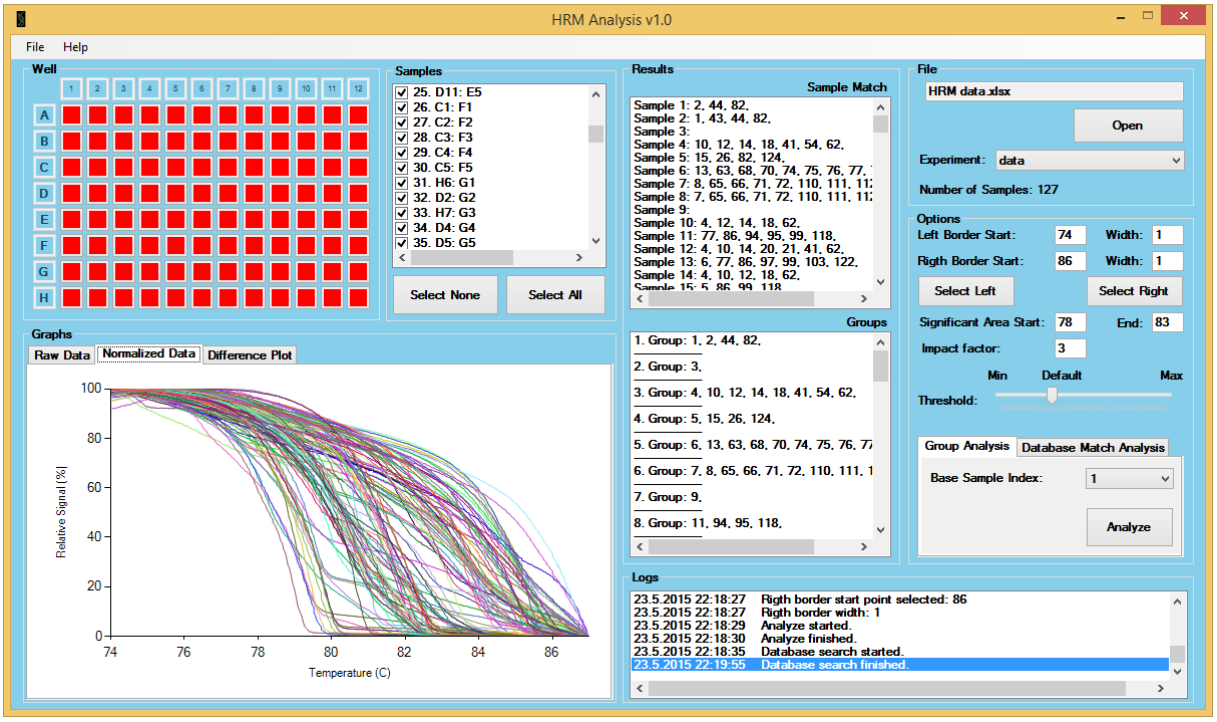

Figure 4.3. Normalized melt curve graph and group analysis of all samples created by HRM software

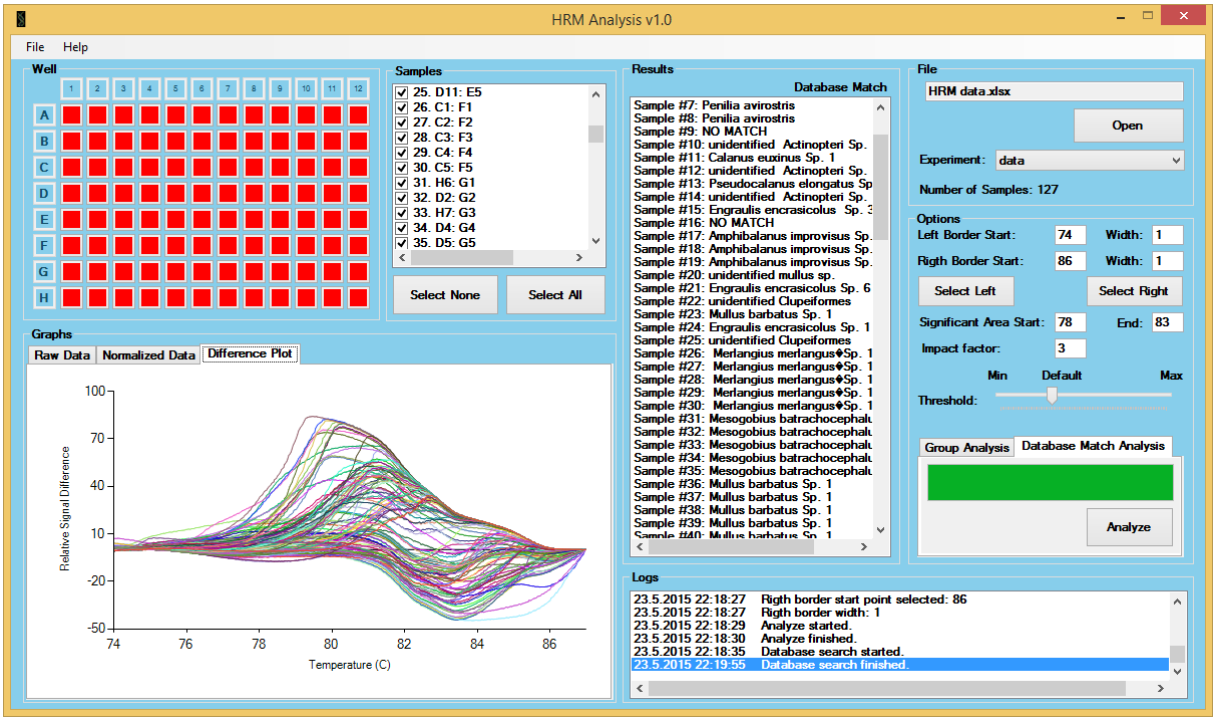

Figure 4.4. Difference plot and database match analysis of all samples created by HRM software

The matching performance of the software depends on the sample quality and type. Therefore, it is a crucial step to adjust the error threshold for the best grouping value. The default threshold is determined as 7% by the empiric conclusions. However, no automatic solution is found to solve error margin adjustments.

In this study, C# programming language is used because it is easy to work with different data types, it has many open source mathematical libraries, it provides qualified user interface and it has a strong support network. However, it costs a significant amount of CPU usage (Gilling et al., 2008). Since the data is relatively small, this problem is not affected system very much. Nevertheless, when the database begins to expand, new solutions might be necessary to increase speed and to decrease cost.

The software is capable of grouping data with each other and matching the data to the created database. The grouping and matching mechanisms are using the difference plot data. When data are grouped with each other, base line can be selected by the user.

However, in matching algorithm the baseline must be the reference data set which is a specific *Homo sapiens* sample. The reasons to select human sample as reference data were produced consistent results and easy to obtain than the Zooplankton organisms.

The main hold-back of the software is that the grouping and matching algorithm is based on the maximum difference points of the lines rather than the profile shape. In some points, the melting curve profile can shift through the temperature or fluorescence axis. Normalization aims to fix fluorescent shift, but not the temperature shift because the temperature shift also has some disadvantages like data loss. Nevertheless, the results indicate acceptable results for beta version of software in the overall.

At the end of the study, there are sequence data, taxonomic data and a HRM data for each sample. Theoretically, each of these data sets refers to other two data sets. However, as discussed before, taxonomic and DNA based identifications were not parallel for some samples. Therefore, in order to diminish this ambiguity, DNA based identification results, phylogenetic tree, taxonomic findings and expectations were taken into account to determine these samples. When the HRM results also added into the equation, this process become harder than it was. Therefore, all the data were grouped according to their quality and accuracy. Firstly, sequence data with very good similarity percentage are accepted highest priority in species identification. Then, the other samples are separated whether they have COI sequence in the NCBI database or not. Blast results are compared to taxonomic data and then their HRM profile compared with the other data sets to determine if their melting curves are similar to taxonomic or sequence based identification. However, the samples with no COI sequence information in the NCBI database is directly accepted according to their taxonomic identification. This concept is applied for vice versa, taxonomically unidentified samples are identified according to sequence data.

After the organisms are identified, a worksheet is created to group all these samples. Each group consists of sequence data, taxonomic data and HRM data. Then, their

HRM profiles are analyzed by the software. In order to create the HRM software database, each of the different HRM profiles is named with a species name that they belong. The results are shown in the Appendix G. Since it was difficult to analyze all the data together, they fragmented into manageable groups. Moreover, these small groups helped to compare haplotypes and the sensitiveness of the software within the same species. Depending on the group, the difference between the haplotypes shows a great variation. For example, this variation is very small for the *Acartia* species, but it is considered high for the *Engraulis encrasicolus*.

5. CONCLUSIONS

The main objective of this study was to create a new and fast method to identify Zooplankton species of Black sea. For this reason, Zooplankton samples were collected from a wide range of Black Sea. Then, the samples were taxonomically identified. Afterwards, their mitochondrial DNAs were extracted and qPCR experiments were carried out. In order to demonstrate the pros and cons of the identification methods, DNA Barcoding was also practiced by using the COI gene. Finally, high resolution melting profiles were used to create a database and software which allows identifying the species instantly.

Taxonomic identification needs expertise, time and effort, but still its reliability depends on the person who performs the work. On the other hand, DNA Barcoding needs less expertise and effort, but it needs more time because of the sequencing step and it is also expensive. However, results more reliable in general terms and the sequence data can offer more knowledge. Yet, the power of this method is limited by the database and Barcoding gene. When there is no entry of a species in the database, taxonomy is a must to solve this problem. Moreover, only one Barcoding gene is not completely reliable to separate all animals. Therefore, it is a fact that making mistakes with DNA Barcoding is inevitable in these conditions. The most accurate method would be performing both techniques, but it would not be feasible.

In conclusion, this new methodology is working as expected and HRM based species identification offers a new page to this area. Even though there are some issues to improve, it can be considered successful for a new method since it is fast, cheap, sensitive and accurate.

6. FUTURE ASPECTS OF THE STUDY

This study covers only a few fish and Zooplankton species in the Black Sea. Therefore, the database is relatively small and easy to handle with basic solutions. In the future, the database could extent with the contributions of scientist in this area. In which case, more sophisticated algorithms and features must be added in to software. Especially, new mathematical approaches can be used to improve matching performance. Moreover, database must cover some other barcoding genes than the COI like ITS, matK and rbcL genes. This study could be an inspiration for developing new methods for identification of species.

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APPENDIX A: SEQUENCES OF THE POTENTIAL ZOOPLANKTON IN BLACK SEA

Table A.1. COI sequences of the potential Zooplankton organisms in Blacksea

Organism	COI sequence
Oithona similis	AGATATTGGAACTCTATATCTTTTAACTGGAGTTTGGGCAGGAATAATT GGAACTAGGATAAGTGTTATTATCCGAATTCAACTTTCGTACCCTACTGGTTTTTTGTGT AATGAGCAGCTTTATAATGTAATGGTTACAGCCCATGCTTTTATTATAATTTTTTTTATA GTAATACCGATCTTAATCGGTTGTTTTGGAAATTGGCTAGTTCCTTTAATAATTGGATCT CCAGATATAGCTTTTCCCCGACTCAACAATATGAGCTATTGACTACTAGTCCCTGCTTTG TTCTTACTACTAGTAGGCTCTATAGTAGAATCTGGAGCTGGTACAGGTTGGACAGTGTA TCCCCCTCTTAGGTCATACATTTTTCATGGAGGCGCTTCTGTGGATTTTACAATTTTCAG GCTGCATTTAGCAGGAGTTTCTTCTCTCCTAGGCGCCGTGAACTTTATTAGAACAGTATT AAATCTTCGTGCATTAGGCATGCTAATAGACCGGATACCTTTATTCCCTTGAGCTGTGTT TATTACAGCTATTCTTTTACTGCTATCACTTCCCGTGTTAGCTGGGGCAATTACGATATT GCTAACAGACCGAAATTTAAACACTTCATTTTACGATCCCATAGGGGGAGGGGATCCT GTCTTGTACCAACACTTATTTTGATTT
Paracalanus parvus	AAGATATTGGAACACTATATTTACTAGCAGGGGCCTGATCTGGTATGAT TGGCACAGGATTAAGAATGATTATTCGTTTAGAATTGGGGCAATCGGGTTCTTTAATTG GCGACGATCAAATTTATAATGTAGTTGTAACAGCCCATGCGTTTATCATAATTTTTTTTA TGGTTATACCTATTTTAATTGGAGGGTTTGGAAATTGACTGGTTCCATTAATACTTGGA GCAGCTGATATAGCGTTCCCTCGAATAAATAATATAAGATTTTGATTTTTAATTCCAGCT TTAATTATATTATTATCTAGTTCTCTCGTAGAAAGAGGAGCAGGAACAGGCTGAACTGT ATATCCTCCTCTATCTAGGAATATTGCTCACGCAGGAAGTTCAGTAGATTTTGCTATTTT TTCATTGCATTTAGCAGGAGTAAGTTCAATTTTAGGTGCGGTTAATTTTATTAGAACATT AGGAAATTTACGAGTGTTTGGAATATTATTAGACCGAATACCTTTATTTGCATGGGCGG TATTAATTACAGCAGTTTTACTATTATTATCTCTACCTGTCTTAGCTGGGGCTATTACTAT ATTATTAACAGATCGAAATTTAAATACAACTTTTTATGATGTTGGGGGTGGTGGGGATC CTATTTTATATCAGCATCTATTTTGATTCTTTGGACATCCTGAAGTCTATATTTTAATTTT ACCTGGGTTTGGATTAATTTCTCATATTGTAGCTCAAGAAAGAGGAAAAAAAGAAACC TTTGGAGTTTTAGGTATAGTCTATGCTATATTACAGCTTGGT
Pseudocalanus elongates	TTAATAGCTGGGGCATGGGCAGGAATAATTGGTACAGGGTTGAGAATG ATTATTCGAATAGAGCTAGGTCAGGCCGGGTCCTTAATCGGGGATGACCAGATTTATA ATGTTGTTGTCACAGCACACGCTTTTATCATAATTTTTTTTATAGTTATACCAATTTTAAT

	TGGAGGGTTTGGTAATTGGTTAGTCCCTCTTATATTAGGGGCAGCAGATATAGCTTTCC
	CACGTATAAATAACATGAGTTTTTGATTTTTAATACCTGCCCTAATTATACTTCTCTCAAG
	TTCTCTAGTTGAAAGAGGCGCAGGCACAGGGTGGACTGTTTACCCTCCGTTATCGAGG
	AATATCGCACACGCAGGAGGGTCTGTAGACTTTGCTATTTTCTCTCTTCATTTAGCGGG
	GGTAAGATCTATCTTAGGTGCGGTAAATTTTATTAGTACTTTAGGTAATTTACGAGTATT
	TGGCATACTTTTAGATCAGATACCATTATTTGCGTGGTCTGTATTAGTAACGGCTATTCT
	TTTACTACTGTCCTTACCCGTCTTAGCTGGAGCTATTACTATATTATTAACAGATCGAAA
	TTTAAATACTTCTTTTTATGAT
Acartia tonsa	ACTTTATATTTATTAGCAGGTATATGATCAGGAATAGTGGGAACAGGAT
	TAAGAATAATTATCCGAATAGAATTAGGACAAGCTGGAAGGCTAATTGGAGATGATCA
	AATTTATAACGTAGTGGTTACAGCTCATGCTTTTATTATAATTTTTTTTATGGTTATACCT
	ATTTTAATTGGAGGATTTGGTAATTGATTAGTTCCTTTAATATTAGGAGCTGCAGACAT
	AGCATTTCCTCGAATAAATAATATAAGATTTTGACTTCTATTACCAGCTTTAATTATATTA
	TTATCTAGGTCGCTAGTAGAAAGAGGTGCAGGTACAGGATGAACCGTTTATCCCCCTTT
	ATCAAGCAATATTGCCCATGCTGGCGCATCAGTAGATTTTGCTATTTTCTCGCTTCACCT
	TGCAGGTGCAAGTTCAATTTTAGGAGCAGTAAATTTTATTTCAACAATTGGTAATTTAC
	GATCTTTTGGAATAGTTCTTGATTTAATACCTTTGTTTGCGTGAGCAGTATTAATTACTG
	CGGTTTTACTATTATTATCTTTGCCTGTTTTAGCAGGTGCAATTACAATATTGTTAACCG
	ACCGAAATTTAAATTCTTCTTTTTATGATGCAAGTGGAGGAGGAGATCCAATTCTT
Acartia clause	ATTCGAATAGAGCTAGGCCAAGCCGGTAAACTAATTGGGGATGATCAA
	ATTTATAATGTAGTGGTAACAGCTCATGCATTTATTATAATTTTCTTTATAGTAATGCCG
	ATTCTAATTGGTGGTTTTGGTAATTGGTTAATTCCTTTAATATTAGGTGCTGCTGATATA
	GCTTTTCCTCGAATGAATAATATAAGATTTTGACTACTTTTACCTGCCTTAGTAATACTTT
	TATCAAGCTCTTTAGTAGAGAGAGGGGCGGGGACGGGATGAACAGTTTACCCTCCTTT
	GTCGAGTAATATTGCTCATGCAGGAGCTTCTGTCGATTTTGCTATTTTCTCCCTTCACCT
	AGCAGGTGCTAGATCGATTTTAGGCGCAGTTAACTTTATTTCAACGATTGGTAATTTAC
	GATCTTTTGGGATAGTAGCTGATCTAATGCCTTTATTCAGGTGGGCAGTAATTATTACA
	GCGGTGTTGTTATTATTATCTTTGCCTGTTTTAGCAGGAGCTATTACTATGCTTTTAACA
	GATCGAAACCTTAATTCTTCATTTTATGATGCAGGAGGGGGAGGAGACCCAATTTTATA
	TCAGCATTTATTTTGATTTTTCGGACATCCTGAAGTTTATATTCTTATCCTTCCTGGGTTT
	GGGCTAATTTCTCATATTGTCTCCCAAGAGAGAGGAAAGAGAGAGACATTTGGGATGC
	TTGGAATAGTTTATGCAATAATATCTATTGGATTACTAGGTTTTGTAGTATGAGCACACC
	ACATGTTTACTGTAGGAATAGATGCAGACACTCGAGCATATTTTACATCTGCTACAATA
	GTAATTGCAGTTCCAACGGGTATTAAAGTGTTCAGGTGATTAGGAACACTTCATGGGG
	TGCGTTTAATTTTTTCTCCTTCAATATTATGATCTTTAGGTTTTATTTTTTTATTTACAGTG
	GGG
Calanus euxinus	ACATTATATTTATTGGCCGGTGCGTACTCAGGAATAATCGGTACGGGAC

	TCAGTATAATTATTCGTCTAGAATTAGGTCAAGCTGGGTCTTTAATTGGAGATGATCAA
	GTATATAACGTTGTAGTAACTGCACACGCATTTATTATAATTTTTTTTATAGTTATGCCTA
	TTTTAATTGGAGGATTTGGAAACTGATTGGTCCCTTTAATATTGGGTGCAGCAGATATG
	GCATTTCCTCGTATAAATAATATAAGATTCTGGTTCTTAATGCCAGCTTTAATTATACTTT
	TGTCAAGATCTCTGGTTGAAAGGGGCGCAGGTACTGGGTGAACCGTGTACCCCCCCCT
	ATCCAGAAATGTAGCCCATGCTGGAGCTTCTGTCGACTTTGCTATTTTTTCGTTACATTT
	AGCTGGGGTGAGATCTATTTTAGGGGCTGTAAATTTTATTAGAACCCTTGGCAATCTTC
	GAGTGTTTGGTATATTGCTTGATCGAATGCCTCTTTTTGCCTGGGCTGTTCTAATTACTG
	CGGTCTTACTTCTTTTATCTCTCCCTGTTTTGGCCGGGGCAATTACAATACTACTTACAG
	ACCGAAACCTAAATACGACATTTTATGATGTAGGGGGCGGGGGAGACCCTATTTTATA
	TCAGCACCTATTT
Penilia avirostris	ATGGTAGGTACTGCTTTAAGAATGCTAATCCGAGCTGAACTAGGACAAT
	GTGGAAGAGTAATTGGTGATGAGCAGATTTACAACGTTGTAGTAACAGCTCATGCCTT
	TGTTATGATTTTCTTTATGGTCATACCAATTTTAATTGGGGGGTTTGGGAACTGATTGGT
	TCCTTTAATGCTCGGGGCTCCTGATATGGCTTTTCCTCGTTTGAATAATTTAAGATTTTG
	GCTTCTGCCTCCTTCTTTAACATTGCTTTTAGTAGGGAGAGCTGTTGAAAGAGGTGCTG
	GTACAGGATGAACCGTTTATCCTCCTTTATCAAGAACAATCGCCCACGCGGGTGCTTCT
	GTAGATCTTARAATCTTCTCCTTGCATTTAGCGGGGATTTCATCAATCCTCGGAGCTGTA
	AACTTTATTACGACAATTGTAAATATACGATCTAAAGGAATAACTTTAGATCGTATTCCC
	CTCTTTGTGTGGGCTGTTGGAATTACTGCTTTATTACTCCTACTTAGACTTCCTGTACTTG
	CAGGAGCTATCACTATGCTTCTGACAGACCGAAATTTAAATACTTCTTTYTTTGATCCTG
	CGGGAGGGGGGGACCCTATTCTTTATCAACACTTGTTCTGATTTTTTGGCCATCCGGAA
	GTTTACATTTTGATCTTGCCTGGGTTTGGTATGATTTCTCATATTATTAGCCACGAAAGG
	GGAAAAAAAGAAGCATTCGGTACCCTGGGTATAATTTATGCTATAATAGCAATTGGTA
	TTTTAGGATTC
Evadne spinifera	GGTATTTGAGCAGGGATAGTAGGAACTGCTTTGAGTATACTAATTCGAG
	CTGAATTAGGACAGGCAGGGAGCTTATTAGGAGATGATCAACTTTATAATGTTATCGTT
	ACCGCTCATGCTTTTATTATGATTTTCTTCATGGTTATACCAATCATGATTGGGGGATTT
	GGGAACTGATTAGTTCCTCTTATGCTCGGGGCCCCAGATATGGCTTTTCCTCGTCTTAAT
	AACCTCAGTTTTTGATTTTTACCTCCAGCACTTACTCTTCTTCTTGCCGGGGGAATGGTA
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	TTTTAGGGGCTATTAACTTCATTACTACGATCGTGAATATACGATCTCAAGGAATGACG
	CTTGATCGAATTCCACTCTTCGTTTGATCAGTAGGGATCACTGCTCTTTTACTTCTTTTAA
	GCTTACCTGTTCTAGCAGGAGCTATTACTATGCTTCTAACGGACCGGAATCTAAACACA
	TCGTTCTTCGATCCTGCAGGGGGAGGGGACCCGATTCTTTACCAACATCTATTC
Evadne nordmanni	GCTGAATTAGGACAGGCAGGGAGCTTATTAGGAGATGATCAACTTTAT

	AATGTTATCGTTACCGCTCATGCTTTTATTATGATTTTCTTCATGGTTATACCAATCATGA
	TTGGGGGATTTGGGAACTGATTAGTTCCTCTTATGCTCGGGGCTCCAGATATGGCTTTT
	CCTCGTCTTAATAACCTCAGTTTTTGGTTTTTACCTCCAGCACTTACTCTTCTTCTTGCCG
	GGGGAATGGTAGAAAACGGAGCAGGGACAGGATGAACTGTCTACCCCCCTCTTTCTGC
	GGGGATTGCGCATGCAGGGGCTTCAGTAGACCTTAGTATTTTCGCTCTTCATCTTGCTG
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	AAGGAATGACGCTTGATCGAATTCCACTCTTCGTTTGATCAGTAGGGATCACTGCTCTT
	TTACTTCTTTTAAGTTTACCTGTTCTAGCAGGAGCTATTACTATGCTTCTAACGGACCGG
	AATCTAAACACATCGTTCTTCGATCCTGCAGGGGGAGGGGATCCGATTCTTTACCAACA
	TCTATTCTGATTTTTTGGT
Pleopis	TGAGCTGGAATAGTAGGAACAGCGTTAAGTATACTAATTCGAGCTGAG
polyphemoides	TTAGGACAGGCGGGAAGTCTAATTGGAGACGACCAACTGTACAACGTAATCGTTACAG
	CTCATGCTTTTGTAATAATTTTCTTCATGGTAATACCTATTATGATTGGAGGATTTGGGA
	ATTGATTAGTTCCTTTAATATTAGGGGCACCTGATATGGCTTTCCCTCGACTTAATAACC
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	TGGTGCATCAGTCGACTTAAGAATTTTCTCTCTTCATTTGGCTGGGATCTCATCAATTTT
	AGGAGCTATTAACTTTATTACTACTATCGTTAATATACGATCTCAAGGAATGACACTTGA
	TCGAATCCCACTATTTGTATGAGCAGTGGGAATTACAGCTCTTCTTTTACTTCTTAGTCT
	ACCAGTATTAGCTGGTGCAATTACAATGCTTCTTACTGACCGTAATCTAAATACGTCATT
	CTTTGATCCAGCTGGGGGTGGAGACCCGATTCTTTACCAACATCTATTTTGATTTTTTGG
	TCAC
Pleurobrachia pileus	ATATGAAGGTGGTTGTTCTGAGTTTATCATAAAGATATCGCTGGTTTAT
	ATTTTTTTTTTTCCATCATTATGGGTTTTATCGGTTTTTTTTACTCGTTGATAATGAGGTTA
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	GCTAGTTCTGGTTGGACGTTATACCCACCTTACTCTTCTTACCCAGGTTCTTCATGATTAT
	CAACTGATTTTATTATATTTTCCTTACATCTTGCAGGTGCTAGTTGAATTCTTTCTTGAAT
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	CTTTGTTTATAGTTGCTCAGCTAACTGTAAGTTTCCTCTTACTGATCTCCTTGCCTGTTCT
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	TTATTTTGCTTCTGCTACCTTAATTATAGCAGTACCCACCGGTATTAAAATCTTCTCTTGG TTATTTACTTTACTATCAGACTCCATAATTTATA
Mnemiopsis leidyi	ATTAGATGATTATTTTCTACTAATCACAAAGATATAGCTTCTCTATATTTT TTTTTTTCTATAATTATGGGTTTTTGTGCTTTTTTCTATTCTTTTGTCATGCGTTTAGCTTT AGTTTGACCTTTTGCTTTTATCGAATCTGGTATTATTTATTTATATTACGTCACTTTACAT GCTGTTTACATGATTTTTTTTTTTGTTATGCCTTTTAGTATAGGTGGTTTATCAAATTTAT TAATTCCATTATGTTTTCATTTGGCTGATATGTGTTTACCTCGTATTAATAATTTATCTTTC TGACTTTTATTTGCTTCTTTTATTATCTCTCTTTTATCCTCTTTTCACTACTATGGTCCAAG TTCAGGATGAACTTTATACCCTCCTTATTCTTCTTATCCTGCTAGTGCCTATTTATCAACT GATTTAATAATTTTCTCTTTACATTTAGCGGGTGCTAGTTCTATATTATCATCCATAAACT TTATAGTTACTGTATTTATTTTACCCATAAATACTTCGTTTTCATTTTTTCAATATCCTTTA TTTATTGTCGCCCAAATTACTGTTTCTTTTCTTCTTTTAATATCATTACCTGTATTAGCTGC AGCTATTACTATGTTACTTTTTGATCGTAATTTTAATACTTCTTTTTTTTCAAATTATCTTG GTGGTGATGCTTTACTTTATCAACATTTATTTTGATTTTTTGGCCATCCAGAAGTTTATGT TTTAATATTACCAGCTTTTGCTATTATTTCTCATGTTTTGTCATTTTTAATTAACAGAAAT GTACCTTTTTCCTATCCTGGCTTAAATATAGCTATAATTAGTATAGGTTTATTAGGTTGTT TAGTTTGAGCCCATCATATGTTTACTTCGGGTATGGATTTAGATACTCGTTTTTATTTTG CTTCAGCTACCTTAATCATAGCTATTCCTACTGGTATTAAAATTTTTTCTTGAATTTTTAC TATTCTTTCTGATACTTTTGTTTT
Aurelia aurita	TCAAGATGATTATTCTCAACTAACCACAAAGATATAGGAACACTATACTT AATATTTGGTGCTTTTTCCGCCATGGTGGGAACTGCCTTCAGTATGATTATAAGACTGG AACTATCAGGCCCAGGATCCATGTTGGGGGACGATCAACTATATAACGTTGTAGTGAC CGCTCATGCTCTTATAATGATTTTCTTTTTCGTAATGCCCGTTTTGATAGGGGGATTTGG AAACTGGCTAGTTCCCCTATATATAGGAGCTCCAGATATGGCCTTTCCAAGGCTTAACA ATATCAGTTTCTGATTATTACCTCCAGCTTTATTACTATTATTAGGGTCTTCCCTTATAGA ACAAGGAGCAGGTACTGGTTGAACCATTTACCCTCCTTTAAGTTCAATACAAGCTCATT CTGGGGGTTCAGTAGATATGGCCATATTTAGTCTTCATTTAGCAGGAGCTTCCTCTATT ATGGGTGCTATTAACTTTATTACCACTATTTTAAATATGAGAGCCCCTGGTATGACCATG GATAGAATACCTTTATTCGTATGATCTGTATTAGTTACTGCAATCTTATTATTGTTGTCCT TACCCGTATTAGCTGGGGCAATTACCATGTTGTTGACTGATAGAAATTTCAACACATCC TTCTTTGACCCTGCTGGAGGAGGAGATCCAATACTATTCCAACATTTATTTTGGTTTTTT GGACACCCAGAAGTGTATATATTGATTCTACCCGGATTTGGAATTGTATCTCAGATAAT ACCAACATTTTCTTCTAAGAAACAAATATTTGGGTATCTAGGAATGGTCTATGCTATGAT AGCTATAGGTATACTTGGATTTATAGTTTGGGCTCACCATATGTTTACAGTTGGTATGG ACGTAGATACTAGAG
Engraulis encrasicolus	GCTGGAATAGTAGGCACGGCCTTAAGCTTGCTCATCCGAGCTGAACTAA GCCAACCAGGTGCCCTTCTTGGGGACGACCAGATCTACAATGTAATCGTTACGGCCCAT

	GCCTTCGTAATGATTTTCTTTATAGTAATACCAATTATGATTGGAGGATTTGGAAACTGA CTTATTCCTCTAATGATCGGAGCCCCCGACATGGCATTCCCACGAATGAACAACATGAG CTTCTGACTCCTTCCCCCCTCTTTCCTTCTGCTCCTAGCTTCTTCAGGAGTTGAGGCTGGA GCCGGAACCGGTTGAACAGTCTACCCTCCCCTTGCCGGCAACCTGGCCCACGCAGGGG CATCAGTTGACCTAACTATTTTCTCACTTCACTTAGCAGGGGTTTCCTCAATTCTTGGGG CAATTAACTTCATCACAACAATTATCAATATGAAACCTGCAGCTATTTCTCAGTATCAAA CACCACTGTTTGTATGAGCTGTACTAATTACAGCTGTTCTTCTCCTACTTTCCCTTCCAGT CCTTGCCGCTGGTATTACAATGCTCCTTACAGACCGAAACCTAAATACAACCTTCTTCGA CCCTGCAGGAGGGGGAGACCCAATCCTTTACCAACACCTA
Trachurus mediterraneus	TTTTATCAGATATTGGGTGCTTGAGCTGGAATAGTAGGAACCGCTTTAA GCCTGCTTATTCGGGCAGAACTAAGCCAACCTGGCGCCCTTCTAGGGGATGACCAAAT TTACAACGTAATTGTTACGGCCCACGCTTTCGTAATAATTTTCTTTATAGTAATGCCAAT TATGATTGGAGGCTTTGGAAACTGACTGATTCCGTTAATGATCGGGGCCCCTGATATAG CCTTCCCTCGAATGAATAACATGAGCTTCTGACTACTCCCTCCCTCCTTCCTTTTGCTTTT AGCCTCTTCAGGGGTTGAAGCCGGGGCCGGAACTGGTTGAACAGTCTATCCCCCACTG GCTGGGAACCTTGCCCACGCCGGAGCGTCCGTAGATTTAACCATCTTCTCCCTTCACCT AGCAGGGGTCTCGTCAATTCTAGGGGCTATTAATTTTATTACCACTATTATTAACATGAA ACCTCCTGCAGTCTCAATATATCAAATCCCACTATTTGTTTGAGCTGTCTTAATTACAGCT GTCCTTCTTCTTCTCTCTCTTCCTGTCCTAGCTGCTGGCATTACAATACTTCTAACAGACC GAAATCTAAATACTGCTTTCTTTGATCCAGCAGGAGGGGGAGACCCAATTCTTTATCAA CACCTATTC
Mullus barbatus	TCTTATATAGTCTTTGGTGCTTGGGCCGGTATAGTAGGAACTGCTCTAA GCCTTCTTATTCGTGCCGAACTCAGCCAGCCCGGTGCTCTCCTAGGAGATGACCAAATT TACAACGTAATCGTTACGGCCCATGCCTTTGTAATAATTTTCTTTATGGTAATACCAATT ATGATTGGAGGGTTCGGCAACTGACTAATTCCATTAATGATTGGAGCCCCCGATATGG CTTTCCCCCGAATGAATAACATGAGCTTCTGGCTCCTTCCGCCCTCATTCCTTCTTCTACT AGCCTCTTCAGGCGTTGAAGCTGGTGCGGGCACCGGTTGGACAGTTTACCCCCCTTTA GCAGGCAACCTAGCACACGCTGGGGCCTCCGTTGACCTAACCATTTTCTCCCTTCATCT GGCAGGCATTTCTTCTATTCTTGGGGCTATTAACTTCATCACCACAATTATTAATATGAA ACCCCCAGCAATTTCACAGTATCAGACCCCCCTGTTTGTGTGGGCCGTTCTCATTACAGC TGTTCTCCTCCTTCTGTCGCTCCCCGTTCTTGCTGCTGGCATCACAATACTTCTTACAGAC CGAAACCTAAACACAACGTTCTTTGATCCCGCTGGCGGAGGGGACCCTATCCTCTATCA ACACCTGTTC
Diplodus annularis	CCTTTATGTTGTATTTGGTGCTTGGGCCGGAATAGTAGGAACTGCCCTA AGCCTGCTCATTCGAGCTGAACTAAGCCAGCCTGGCGCTCTCCTTGGAGACGACCAGA TTTATAATGTAATTGTTACAGCACATGCATTTGTAATAATTTTCTTTATAGTAATACCAAT CATGATTGGAGGCTTTGGAAACTGATTAATTCCCCTTATGATCGGTGCCCCCGATATAG

	CATTCCCCCGAATAAATAATATGAGCTTCTGATTACTCCCCCCATCGTTCCTTCTCCTGCT AGCTTCTTCCGGAGTTGAAGCTGGGGCTGGGACCGGGTGAACAGTTTACCCGCCTCTG GCAGGAAACCTTGCCCACGCAGGTGCATCAGTTGACTTAACCATTTTCTCCCTCCCCCTA GCCGGGATCTCATCTATTCTTGGTGCTATTAACTTCATCACCACAATTATTAACATGAAA CCTCCCGCTATTTCGCAATATCAAACACCGCTATTTGTATGAGCTGTCCTAATTACTGCC GTATTACTTCTTCTATCTCTTCCAGTCCTTGCCGCAGGCATTACAATACTCCTCACAGATC GAAACCTAAACACCACTTTCTTCGACCCAGCAGGAGGGGGAGACCCAATTCTCTACCA ACATCTATTT
Scolelepis squamata	GGAACCCTGTATTTCATACTTGGTATGTGATCAGGTCTCTTAGGGACAT CTATAAGACTTCTTATTCGGGCCGAGTTAGGTCAACCTGGCTCCCTTCTTGGAAGGGAC CAGCTATACAACACTATTGTAACTGCACATGCATTTTTAATAATTTTCTTTCTAGTTATAC CTACATTTATTGGTGGATTTGGGAATTGACTTCTCCCACTTATACTAGGTGCACCAGATA TAGCATTCCCCCGTTTAAATAACATAAGATTCTGACTTCTTCCTCCTTCCTTAGCCCTTCT TCTTGCCTCCGCAGCAGTTGAAAAAGGCGTAGGAACAGGGTGAACTGTCTACCCTCCC CTCTCAAGAAACTTAGCTCACGCAGGTCCTTCTGTAGACCTTGCAATTTTCTCTCTACAC CTTGCGGGGGTCTCCTCTATTCTTGGGGCTCTAAATTTTATTACCACTGTAGTCAATATG CGGTGAGATGGCCTTCGTCTAGAAAATATCTCACTCTTTGTTTGAGCCGTAACAATTAC CGCAATTCTTTTATTACTCTCCCTTCCTGTCCTTGCGGGAGCGATTACCATACTTTTAACA GACCGTAATTTAAATACTTCTTTCTTTGATCCTGCGGGAGGGGGGGACCCTATTCTTTA CCAGCATCTATTT
Alitta succinea	GTATATGATCAGGTCTTCTAGGAACCTCTATAAGACTCCTGATTCGAGC AGAACTTGGTCAACCTGGCGCCCTACTTGGAAGAGACCAGTTATATAATACAATCGTCA CTGCTCACGCCTTCTTAATGATTTTCTTCCTCGTTATACCAGTTATAATCGGGGGATTTG GTAACTGATTGGTACCTTTAATATTAGGAGCCCCAGATATAGCTTTTCCTCGACTTAACA ACATGAGTTTCTGATTATTACCTCCATCTCTAATTCTTTTACTATCCAGGGCTGCAGTAG AAAAAGGAGTTGGTACAGGATGAACTGTGTACCCTCCCCTTTCTAGTAATATTGCCCAC GCCGGCCCATCAGTAGATTTAGCAATTTTCTCTCTCCACCTTGCAGGAGTTTCATCCATC ATAGGAGCTCTTAACTTCATTACAACAGTTATTAATATACGATCTAAGGGATTACGCTTA GAACGAGTACCCCTATTCGTCTGATCTGTAGTGATTACCGCGG
Nephtys hombergii	CTTCTAGCAACCTCAATAAGACTTCTTATCCGGGCTGAATTAGGACAACC CGGTGCTTTATTAGGAAGAGATCAGCTTTATAATACAATTGTTACTGCTCACGCTTTCTT AATAATTTTCTTCTTAGTAATACCAGTAATAATCGGAGGGTTTGGAAACTGACTTGTTCC ATTAATACTAGGAGCCCCTGATATAGCTTTCCCTCGTTTAAATAATATATCTTTCTGACTT CTTCCCCCTTCTTTAATTCTTCTTGTTATATCCGCAGCTGTAGAAAAAGGAGTCGGGACC GGTTGAACCGTTTACCCCCCTCTATCTAGAAACATTGCTCATGCCGGAGCAAGAGTTGA CCTTGCTATTTTCTCTCTTCACTTAGCTGGAGCCTCTTCAATTTTAGGGGCCCTAAATTTC ATTACTACAGTTATAAACATACGATGAAAAGGACTACGATTAGAACGTGTTCCTTTATT

	CGTATGAGCAGTTAAAATTACTGCTATTCTTCTACTTTTATCCCTTCCAGTTCTTGCGGG GGCAATTACAATACTTCTTACAGACCGAAACCTAAACACTTCTTTCTTCGACCCTGCAGG GGGAGGAGATCC
Pholoe synophthalmica	GGCACACTATATTTTATTTTTGGAACCTGATCTGGCTTATTAGGCACCTC CATAAGGATGCTTATTCGTGCTGAATTAGGACAACCCGGGTCTTTACTAGGAAGAGAT CAGCTTTATAATACAATTGTGACAGCACATGCGTTTCTAATAATTTTTTTCTTAGTCATAC CTATCTTAGTAGGAGGGTTCGGTAACTGACTTATTCCCCTTATATTAGGAGCTCCTGAT ATAGCGTTCCCCCGTTTAAACAACATAAGATTCTGGTTATTGCCCCCCTCGCTAATTCTT TTATTAAGATCCAGTGCAGTTGAAAAAGGGGTTGGGACTGGATGAACAGTCTACCCCC CTCTAGCAGCAAACATTGCCCACGCTGGCCTTCAGTTGACCTAGCTATTTTTTCACTTCA TATTGCAGGAGTTTCATCAATTCTAGGGGCATTAAACTTCATCACCACAGTCCTTAATAT ACGATATAAAGGACTACGATTAGAACGGGTACCTTTATTTGTTTGAGCTGCTAAAGTAA CCGCCATTCTATTACTTCTGAGGCTCCCTGTATTAGCTGGTGCAATTACCATACTACTAA CAGACCGTAATTTAAACACTGCTTTCTTTGACCCTGCGGGGGGAGGAGACCCAATTCTC TACCAACACTTATTT
Phyllodocidae	ACTTTATATATAATTTTTGGGATTTGATCTGGGCTTCTTGGAACTTCTATA AGAATGTTAATTCGTGCTGAGTTGGGGCAGCCCGGCTCTTTGTTAGGAAGGGATCAGC TTTATAATACAATTGTTACTGCACATGCTTTTTTAATAATTTTTTTTTTTGGTTATGCCTGTT ATAATTGGAGGGTTTGGAAATTGGTTAGTTCCTTTAATGCTTGGAGCTCCTGATATAGC TTTTCCTCGTTTAAATAATATAAGGTTTTGGTTACTTCCACCTTCTCTCATTATACTTTTAG GGTCTGCTGCAGTAGAGCAGGGTGCTGGTACTGGCTGAACAGTTTATCCTCCCTTATCT AGCAATGTTGCTCATTCAGGTCCTTCAGTTGATTTAGCTATTTTTTCTTTACACTTAGCAG GGGTGTCTTCTATTCTTGCTTCAATTAATTTTATTACCACAGCAATAAATATGCGTTCTA GAGGTCTACGATTAGAGCGGGTTCCTTTATTTGTCTGGTCAGTTGCTATTACTGCTCTGC TTCTTTTACTATCACTTCCTGTTCTAGCAGGTGCTATTACTATATTACTTACTGATCGTAA TTTAAATACTTCTTTTTTTGACCCTGCTGGGGGTGGTGATCCTATTTTATATCAGCATCT
Mytilus galloprovincialis	TCTTTATCTATATAGGGGGGTCTGAGGAGGTTTGTTCGGGGCAAGGTTA AGTCTGATAATTCGGATACAGTTAGGGCATCCTGGAGTATTTTTAAAAAGTGACTGGTT TTATAATGTGGTTGTTACAACACATGCCTTAATAATAATTTTCTTTGCTGTAATACCGAT CCTAATCGGAGCTTTTGGTAATTGGCTGATTCCTCTATTAGTAGGTGGTAAAGATATAA TTTATCCGCGGATAAATAATTTGAGTTATTGGTTATCTCCTAATGCGCTATATTTACTTAT ATTATCTTTTAGAACGGATAAAGGGGTAGGTGCTGGATGGACTATTTACCCGCCATTGT CTGTATACCCTTATCATAGCGGGCCGAGGATAGATGTTCTTATTGTGTCCTTGCATTTAG CTGGGTTAAGTTCTTTGGTGGGTGCTATTAATTTTGCCAGTACCAACAAAAACATACCA GTTTTAGAGATAAAAGGAGAACGAGCTGAGCTTTATGTCCTATGGATTAGAGTTACTG CCGTATTGCTAATTATTTCTATTCCGGTTTTAGGAGGGGGTATCACAATAATTCTGTTTG ATCGGAATTTTAACACAACATTTTTTGATCCAGCAGGAGGGGGTGACCCTGTCTTGTTT

	CAACATTTGTTC
Scapharca inaequivalvis	TCCGCGGTTAAATAATTTCAGTTACTGAATTTTACCAGGCGCTTTATTTA TAGTAATAATATCTGCCTTAATCGAGGGGGGGGTGGGTACTGGCTGGACGTTATATCC TCCTTTATCAAGGTGAATTTTTCATAGAAGTCCAGCTTTAGATATAGTAATTCTTTCTCTT CACATTGCAGGATTTGGGTCAATAATAAGTTCTGTAAATTTTATAAGTACAATAATCAC AAGTCGGTTTTTTGTTTTAATTCCTGAGCGGATACCTGTTTTTTGTTGGTCGATATTTGT AACGTCTTGGTTACTATTGTTCTCTCTGCCAGTGTTGGCTGGAGGGTTAACTATGTTATT AACGGATCGTCATGTAAATAGCTCTTTTTTTCGTCCTCAAGGTGGTGGGGATCCTTTATT ATTTCAACATTTGTTTTGATTTTTTGGTCATCCGGAAGTTTATGTTCTAATTCTCCCCGGG TTCGGGTTAATTAGTCATACAATTATTAAGAGAGGCGGCAAGTTGCGAGTTTTTGGCCT CGCAGGAATGGTATATGCTATACAATCTATTGGAGTATTAGGATTCGTTGTGTGGGCTC ACCATATATTTACAGTAGGAATAGACGTTGATAGTCGTGCCTATTTTACTGGAGCAACG ATGGTAATTGCCATTCCTACAGGAATTAAAGTTTTCAGATGATTAGCAACTCTTCACGG AAGGGTGCTACTTCGGTATACACCTAGGTTTTGTTGAGTACTGGGGTTTTTATTTTTGTT TACTATAGGCGGCCTAACTGGTGTAATTCTATCACATGGTA
Hydrobia acuta	ATTTTATTTGGTATGTGGTCTGGGTTAGTAGGTACAGCACTAAGTTTGTT AATTCGTGCTGAACTAGGTCAGCCGGTGCGCTTTTGGGTGATGATCAGCTTTATAACGT AATTGTTACTGCTCATGCCTTTGTTATGATTTTTTTTCTTGTAATGCCTATAATAATTGGT GGCTTTGGAAATTGATTAGTGCCTTTAATACTTGGTGCTCCAGATATAGCTTTTCCTCGG CTTAATAACATAAGTTTCTGACTTTTACCTCCTGCTTTGCTATTATTACTTTCTTCGGCAG CTGTAGAGAGAGGAGCGGGGACAGGATGAACCGTGTATCCCCCATTATCTAGTAACAT TGCTCACGCGGGGGGGTCTGTAGATTTAGCTATTTTTTCTCTCCACTTAGCGGGTGTTTC TTCTATTCTTGGGGCTGTAAATTTTATTACAACTATCATTAATATACGGTGACGAGGAAT GCAGTTTGAGCGGCTTCCGTTGTTCGTATGATCTGTAAAAATTACTGCCATTCTATTATT ACTATCTTTACCTGTCTTAGCTGGTGCTATTACTATGCTTTTAACGGATCGAAATTTTAAT ACTGCATTTTTCGACCCAGCAGGAGGTGGAGATCCTATTTTATAC
Retusa sp.	GACTTTATATATAATTTTTGGAATATGATGTGGTCTTGTAGGAAGAGGG TTAAGGTTACTAATTCGGTTCGAGCTAGGAAATGTTTCAGCTTTTTTAGAGGATGATCA TTTTTACAATGTTATGGTCACAGCCCATGTGTTTGTAATAATTTTTTTTATAGTTATACCC TTAATAATTGGGGGGTTTGGGAATTGAATAGTTCCTTTATTAATTGGGGCTCCTGATAT AAGGTTTCCTCGGATAAATAATATAAGATTTTGGCTTCTTCCTCCTTCTTTTATCTTATTA TTAGTATCAAGAATAATTGAAGGAGGGGCAGGGACAGGATGAACTGTTTATCCTCCTC TATCAGGGCCGATTGCACACGGTTCTACATCTGTAGATTTAGTTATTTTTTCCCTACATC TTGCTGGAATATCATCAATTTTAGGGGCTATTAATTTTATTACTACTATCATTAATATAC GTTCCCCAGGGATTACATTTGAACGTTTAAGTTTATTTGTTTGGTCAGTTTTTGTGACAA CATTAAAGATGTTACTTTTATTACCTGAAACGGCGTGAACTATTATACAACTTTTTACAC ATTGAAATTTAAATACTAGGTTTTTTGATCCAGCAGGAGGGGGGGACCCAATCTTATAT

	CAACATTTATTT
Haminoea navicula	AATCATAAAGATATTGGAACACTATATATAATCTTTGGNATGTGATGTG
	GTCTAGTAGGTACGGGACTTAGTCTGCTAATTCGGTTCGAACTAGGAACAGCATCAGC
	TTTCCTTGGAGATGATCACTTTTATAATGTAATTGTTACGGCTCATGCCTTTGTAATAAT
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	TACTATGCTTTTAACTGATCGAAACTTTAATACGAGGTTCTTTGATCCGGCAGGAGGTG
	GTGACCCTATTCTCTACCAACACCTGTTTTGATTTTTTGGTCACCCTGAA
Tergipes tergipes	TACTTTGTACATATTTTTAGGTATGTGATGCGGCCTAGTTGGTACTGGGT
	TAAGTTTATTAATTCGGTTTGAATTAGGTACTGCTGGTGCTTTGCTAGGTGATGATCATC
	TTTACAATGTAATTGTAACTGCCCATGCTTTTGTTATAATTTTTTTCATGGTTATGCCTTT
	AATAATTGGGGGTTTTGGTAATTGGATAGTTCCTTTACTAATTGGTGCTCCTGATATAA
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	CAGGTATGTCTTCTCTGTTGGGGGCTATTAACTTTATTACTACTATTTTTAATATGCGAT
	CTCCGGAGATAACGTGAGACCGGTTAAGGTTGTTTGTGTGGTCTGTTCTTGTAACTGCT
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	CTTTTTAACTTTTTAAAGAAGTTACAATGTAAAAATTAGTAATAATTGTTTTAAATAAGT
	TTATTAGTTTAATTACTATACTTTTTTATTTTTAAATAGTTTTAAGGAACTCGGCAAAAAT
	AGACTTGGACTGTTTAACAAAAACATAGCCACTTGAATATATTTGGGTTTAACCTGCCC
	AATGTAACTAATATGAATGCCCGCGGTACCTTGCCCGTGCTAAGGTAGCGTAATCAGTT
	GGCTTTTAAATGGAGTCAGGTATGAATGGGATAACTGGGTCTAGCTGTCTCAGAAATA
	TTTTTTTGAATTTACTGTTTAAGTGAAAAAGCTTAAATTTTATAAAGGGACGAGAAGAC
	CCTTGGAATTTTTTTTAATTTAGGATTATTCCTGATGTTATTTTGTTGGGGCGACATAGA
	AATAGTTAAAACTTTCTTTATAACATGAGTCGGAATTTTCAGGAAAAGATAAATTACCC
	AAGGGATAACAGCATAATTCTATAAATGAGTTTGTGACCTCGATGTTGGACTAGGAAG
	CTGGCAGGTTAGCTGCTTGTCGTGCGAATTCTGTTCGAATTTTAACTCCT
Upogebia	TACATTATATTTTATATTTGGAGCATGAGCCGGAATAGTGGGAACTTCA
	CTAAGATTGGTTATTCGAGCAGAATTAGGTCAACCTGGAAGATTAATTGGAGATGACC
	AAATTTATAATGTTGTTGTTACCGCCCACGCCTTCCTTATAATTTTTTTTATAGTTATACC
	TATTATAATCGGAGGTTTTGGAAATTGACTAGTACCCCTTATATTAGGAGCTCCTGATAT
	AGCTTTTCCCCGTATAAATAATATAAGATTTTGACTTTTACCTCCATCATTAACACTACTT
	TTAATAAGAGGAATAGTAGAAAGAGGTGTTGGGACAGGATGAACAGTTTACCCTCCTT
	TATTAGCAGCCATTTCCCACGCAGGTGCTTCCGTTGATATGGGTATTTTTTCTCTTCATTT
	AGCAGGTGTGTCATCAATTTTAGGAGCAGTAAATTTTATTACCACAGTTATTAATATAC
	GATCTGTAGGGATAACTATGGATCGAATACCCCTATTTGTATGATCAGTATTTATTACTG
	CTATTTTATTACTTCTATCTCTACCAGTTTTAGCTGGAGCTATTACTATACTTTTAACAGA
	TCAAAATTTAAATACCTCATTTTTTGACCCAGCTGGAGGAGGAGATCCTATTTTATACCA
	ACATTTATTT
Xantho poressa	TGTTCTCTACCAACACTTATTTTGATTTTTTGGCCATCCAGAGGTTTATAT
	TCTAATTCTTCCCGCCTTTGGTATAATCTCCCATATTGTAAGACAAGAATCAGGGAAAA
	AAGAATCTTTCGGTACATTAGGGATGATTTACGCTATGTTGGCTATTGGTGTTCTAGGA
	TTCGTCGTATGAGCTCATCACATGTTTACAGTAGGTATGGATGTGGATACCCGGGCGTA
	CTTTACTTCTGCTACTATAATTATTGCGGTCCCCACCGGTATTAAAATTTTCAGATGATT
	AAGAACCTTACACGGCACTCAAATTTCTTACAGACCTTCGTTACTTTGAGCATTAGGGTT

	TATCTTCCTCTTCACAGTTGGAGGTTTAACCGGGGTCGTACTAGCTAACTCTTCTATTGA CATTATCCTTCATGATACATACTATGTCGTCGCTCATTTCCATTATGTACTGTCTATAGGA GCTGTATTTGGAATCTTCGCTGGAATTGCCCATTGATTCTCATTATTCACTGGCCTTTCCT TAAATCCTAAATGGTTAAAAATTCATTTCTTCGTCATGTTTGTTGGTGTTAATACAACCT TCTTTCCTCAGCATTTCCTAGGACTGAACGGTATACCTCGGCGTTACTCC
Rhithropanopeus harrisi	TACATTATATTTTATTTTTGGAGCATGAGCTGGTATAGTAGGAACCTCAT TAAGTTTAATTATTCGAGCTGAACTAGGTCAACCTGGTACCCTCATTGGTAATGACCAA ATTTACAATGTTGTAGTAACAGCTCACGCCTTTGTAATAATCTTTTTCATAGTTATACCCA TTATAATTGGAGGATTTGGTAATTGACTAGTTCCATTAATATTAGGAGCCCCTGATATA GCATTTCCTCGTATAAATAATATAAGATTCTGACTTTTACCACCATCACTTACACTCCTCC TAATAAGAGGAATAGTAGAAAGAGGAGTTGGAACAGGATGAACTGTATATCCTCCTTT AGCTGCTGCTATTGCTCATGCAGGAGCCTCCGTTGATATAGGAATCTTCTCCTTACATTT AGCAGGTGTTTCTTCTATTTTAGGTGCCGTTAATTTTATAACAACCGTAATTAATATACG ATCATTTGGTATAACTATAGACCAAATACCATTATTTGTTTGAGCAGTATTTATTACTGC TATTTTATTACTTTTATCTTTACCTGTATTAGCTGGAGCCATTACTATACTTTTAACTGAT CGTAATTTAAATACCTCATTTTTCGATCCTGCTGGAGGAGGAGACCCTATTTTATACCAA CATTTATTT
Crangon crangon	ATTTTAATCCTGCCTGCCTTTGGAATAATTTCTCATATTATTAGACAAGA AAGAGGTAAAAAAGAAGCCTTTGGTACCCTTGGTATAATTTATGCTATAATAGCAATTG GGGTTTTAGGTTTTGTAGTATGAGCACATCATATATTCACAGTAGGTATAGATGTGGAC ACACGAGCATACTTCACTTCAGCAACTATAATTATTGCTGTCCCTACAGGTATTAAAATT TTCAGATGACTAGGTACTCTTCATGGTACTCAACTTTTTTATAGACCTTCATTAATATGA GCTCTTGGATTTGTTTTCCTTTTCACAGTTGGAGGTTTAACAGGAGTAGTTCTAGCTAAT TCATCAATTGATATTCTCTTACATGATACATATTATGTAGTAGCACATTTCCATTATGTAT TATCTATAGGGGCGGTGTTTGGTATTTTTGCAGGATTAATTCATTGATTCCCTTTATTTA CAGGCCTATCATTAAATGATAAATTATTAAAAATTCATTTTATCACTATATTTGTAGGAG TAAATATTACTTTCTTCCCTC
Palaemon elegans	ATTTTATTTTCGGAGCTTGAGCAGGAATAGTAGGGACTTCTCTAAGACT TTTAATTCGAGCTGAATTAGGTCAACCTGGTAGGTTAATCGGAAATGACCAAATTTATA ATGTTATTGTTACCGCCCACGCTTTCGTTATAATCTTTTTTATGGTTATGCCAATTATAAT TGGCGGGTTTGGAAATTGACTGGTACCATTAATGCTAGGAGCCCCTGATATGGCTTTTC CACGAATAAATAATATAAGGTTTTGACTTTTACCCCCTTCCTTAACTCTCCTTCTTTCTAG AGGGATGGTTGAAAGGGGAGTGGGAACAGGATGAACTGTTTACCCTCCTCTAGCGAG AGGATTAGGACATGCTGGCGCTTCTGTAGATCTTGGTATTTTCTCCCTTCATTTAGCAG GAATCTCTTCCATCCTAGGAGCAGTTAACTTTATTACTACTGTAATCAATATACGAGCTC CAGGTATAACTATAGATCGAACTCCTCTTTTCGTGTGGGCTGTTTTTCTAACAGCTATTC TTCTTTTACTATCCTTACCAGTTTTAGCAGGGGCTATCACCATGCTCCTTACTGACCGTA

	ATTTAAATACTTCATTCTTTGATCCTGCTGGAGGGGGTGACCC
Athanas nitescens	CGCTATATTTTATTTTCGGAGCCTGAGCCGGGATATTAGGCACATCCCTC AGACTATTAATTCGAGCGGAGCTAGGACAACCAGGAAGCCTTATTGGAAATGATCAAA TTTATAATGTAATTGTTACCGCCCATGCCTTTATTATGATTTTTTTTATAGTCATACCTATT ATAATTGGAGGCTTCGGTAATTGACTGATCCCACTTATATTAGGTGCCCCGGACATAGC CTTCCCCCGGATGAATAACATAAGGTTCTGGCTATTACCACCATCTCTCACGCTACTACT ATCTAGAGGAATAGTAGAAAACGGGGTTGGAACAGGATGAACTGTATACCCTCCTCTG TCAACCAATATCGCACATGCAGGGGCCTCGGTGGACCTTGGTATTTTCTCTCTTCACCT GGCAGGAGTCTCTTCGATCCTAGGAGCTATTAACTTTATAACTACTGTTGCTAATATACA ACCAGGTGGTGTAACTTTTGACCAACTATCTCTTTTCACCTGATCTGTCTTTCTCACAGC CATTTTACTCCTACTCTCCCTACCAGTCTTAGCGGGAGCAATTACAATACTTCTTACAGA CCGAAACCTCAACACATCTTTCTTTGATCCTGCAGGAGGAGGAGACCCTATTCTCTACC AACACTTATT
Alpheus sp.	GAAGTTTATATTCTAATTCTACCAGCTTTCGGTATAATCTCCCACATTATT AACCAAGAGTCTGGTAAAAAAGAAGCATTTGGAACCCTAGGTATAATCTACGCCATAG CAGCAATTGGAATCCTAGGATTTGTAGTATGAGCCCACCACATATTTACAGTCGGCATG GACGTTGACACGCGGGCCTACTTCACATCAGCCACTATAATTATTGCAGTTCCCACTGG AATTAAAATTTTCAGGTGGCTGGGCACCCTCCATGGAACACAATTCACCTACAGACCGT CCCTCCTATGGGCCCTAGGGTTTGTATTCTTATTCACAATGGGAGGACTAACTGGCGTG GTCCTAGCTAACTCTTCTATCGATATCATCCTCCACGACACGTACTATGTCGTAGCACAC TTCCACTACGTCTTATCAATAGGAGCAGTGTTTGGAATTTTCGCCGGAATCGCCCACTG GTTCCCCCTATTTACCGGCCTATCCCTCAACCCCCAGTGACTTAAAATACACTTCTTTACT ATATTTATTGGGGTAAACATTACATTCTTCCCC
Diogenes pugilator	GGCTCTTCACTCAGGGTGCTAGTGCGCCTAGAGTTAGGTCAGCCAGGG GGCTTAATTGGAGACGATCAGATCTACAATGTAATTGTTACAGCTCACGCTTTCGTTAT AATTTTCTTTATAGTTATACCTATTATAATTGGGGGGTTTGGAAATTGGCTGGTACCTTT AATGTTAGGTGCGCCAGATATGGCTTTCCCACGTATAAACAATATAAGGTTCTGGTTGT TACCTCCTTCTTTAACCCTTCTCCTAAGTAGTGGTTTAGTTGAGAGAGGGGTAGGGACA GGGTGAACTGTTTATCCTCCCTTAGCGTCTGGTATTGCTCATGCCGGGGCTAGGGTTGA CCTGGGTATTTTTTCTTTACATTTAGCTGGGGCTTCTTCTATTCTAGGGGCTGTTAATTTT ATCTCTACTGTGATTAATATACGCAGGCCTGGTATAACTTGGGATCGGCTGCCTTTGTT CGTGTGGTCTGTCTTTATTACAGCGGTGTTACTGCTATTGTCGCTTCCGGTCCTCGCCGG GGCTATTACTATACTTTTAACAGATCGGAATTTAAATACTACTTTTTTTGACCCAACTGG GGGAGGGGATCCGATCTTGTACCAGCATCTATTTTGATTCTTTGG
Pachygrapsus marmoratus	TGGTATAGTTGGAACCTCTTTAAGTTTAATCATTCGAGCAGAACTTAGAC AACCAGGTAGTTTAATTGGTAATGATCAAATCTATAATGTTGTTGTTACAGCTCATGCTT TTGTTATAATCTTTTTTATAGTTATACCGATTATAATTGGTGGATTTGGAAACTGGCTTG

	TTCCTTTGATATTAGGAGCCCCAGATATAGCTTTTCCGCGAATAAATAATATAAGATTTT GACTTTTACCTCCCTCCTTATCTCTCTTACTTACAAGAAGAATAGTGGAAAGTGGTGTGG GCACCGGATGAACTGTCTACCCACCCCTCGCCGCCGCTATCGCTCATGCCGGAGCCTCA GTTGATTTAGGAATTTTTTCTCTTCACCTAGCGGGTGTTTCCTCAATCCTAGGAGCCGTT AATTTTATAACTACTGTTATCAACATACGCTCTTATGGTATGACAATAGACCAAATACCA TTGTTTGTTTGAGCAGTCTTTATTACTGCTATCCTTCTCTTGCTTTCCTTACCTGTATTAGC AGGCGCTATTACTATATTATTAACTGACCGTAACTTAAATACTTCATTCTTTGATCCTGCT GGGGGGGGTGACCCTGTCCTCTACCAACATTTATTC
Macropodia	CACTTTATATTTTATTTTTGGAAGGTGATCAGGAATAGTAGGTACTGCCT TAAGAATGATTATCCGAACCGAACTTGGTCAACCAGGAACATTTATTGGAAATGACCA AATTTATAACGTTATTGTTACAGCCCACGCTTTTGTAATAATTTTTTTTATAGTAATACCA ATTATGATTGGAGGATTTGGAAATTGATTAGTTCCTCTTATACTAGGAGCCCCTGATAT GGCTTTCCCTCGAATAAATAATATAAGATTTTGATTATTACCCCCAGCTTTAACCTTACT ACTTATAAGAAGAATAGTAGAAAGAGGAGTAGGAACTGGTTGAACAGTTTATCCTCCT TTATCAAGATCTATTGCTCACGCAGGAGCTTCAGTTGACATAGGAATTTTCTCTCTTCAT TTAGCTGGTGTTTCTTCAATTCTAGGAGCTATTAATTTTATTACTACAGTAATTAATATAC GATCATACGGGATAAATTTAGATCAAATACCTTTATTTGTATGATCAGTATTTATTACTG CTATTTTACTTCTTTTATCACTTCCAGTTCTTGCAGGAGCAATTACAATATTACTTACAGA CCGAAATTTAAATACTTCATTCTTCGATCCAAGAGGAGGAGGCGATCCTATTCTTTATCA ACATTTATTC
Balanus	TTGGTTCTACTCTATCTTTTATTAGAGCCTTAGGATTTATTTATATTATTT GAGAAGCTATAGTATCACAACGACCTACAATTTTTAGTCCAAATTTATCTTCTAATTTAG AATGAGTTCATACTACTCCTCCTCACTATCATAGTTATGATGAACTTCCACAATTTACTAT TCGATAATTCTATAAAATAAATATAGTAGATTTTTAATCTACCTAAAGTTTAATACTTAA GAATTTTAACCTTTTTAAGGTTACGATAGGTGAATCTGTAAGACCTCGAACAACCTTCCT AGTGGTATTCCTTTGTTACACTTATATCTCCGATGACCAGCCGGCCGGCTTTAGTATAAT GGTCAAAGGAGCTATAGTTGTAACAAAGGGATACTGCTAAGAAGGTTGTACGAGGTTT TATAAGTTCACCCAGATTGTTACGATATTTATATATTTATTATATATCTATGTTTAACCTA CAAGAAATAAACCTTTACTAAGGTCTTGTGGTCTGAAATGGCAGATTAGTGCTGTAGAT TTAAGATCTACCCAAAAAGGTTTAAGTCCTTTTTTCAGAATTTAATGTCAACATGATCAC AATTAAGTTTTCAAGATAGAGCTTCCCCATTAATAGAAGAATTAATTATATTCCACGACC ACGCAATATTAGTTTTAACTTTAGTAACAACTTTAGTTGCTTATATTATTTTAACAATATT TAGAAATAAATTTGTAGACCGATTCCTTTTAGAAGGGCATTTAATTGAAGTAATTTGAA CAG
Phoronis	AAAGATATTGGAACATTATATTTAATTTTCGGAGTGTGAACAGGATTAG TAGGAACTGGGCTAAGAGCTTTAATTCGATTAGAATTAGGTCAACCTGGGACTCTTTTA GGAGATGATCAATTGTATAATGTAATTGTTACAGCTCATGCTTTTGTTATAATTTTTTTT

	ATAGTAATGCCTGTCATAATAGGGGGGTTTGGAAACTGGCTGGTTCCTTTAATAATTGG AGCTCCAGATATAGCTTTTCCACGACTAAATAATATAAGTTTTTGATTACTACCCCCTTCT CTTATACTCTTGTTAAGCTCTGCTGCAGTAGAGAGAGGAGTTGGAACTGGCTGAACAG TCTACCCTCCTTTAGCAGGAAATATTGCACATAGAGGAGGTTCTGTAGACTTAGCCATT TTTTCTTTACATCTAGCAGGAATTTCTTCTATTTTAGGTTCAATTAACTTTATTACGACAG TAATTAATATACGATGAGAGGGCTACCAATTAGAGCGTGTTCCGCTGTTTGTGTGGTCT GTCAAGTTTACTGCGATTTTACTTGTTTTATCTTTACCTGTACTGGCTGGGGCAATTACT ATATTATTAACTGACCGGAACTTTAATACTTCTTTTTTTGACCCAGCAGGAGGGGGGGA TCCAATTTTATATCAGC
Verruca	TTGGTTCTACTCTATCTTTTATTAGAGCCTTAGGATTTATTTATATTATTT GAGAAGCTATAGTATCACAACGACCTACAATTTTTAGTCCAAATTTATCTTCTAATTTAG AATGAGTTCATACTACTCCTCCTCACTATCATAGTTATGATGAACTTCCACAATTTACTAT TCGATAATTCTATAAAATAAATATAGTAGATTTTTAATCTACCTAAAGTTTAATACTTAA GAATTTTAACCTTTTTAAGGTTACGATAGGTGAATCTGTAAGACCTCGAACAACCTTCCT AGTGGTATTCCTTTGTTACACTTATATCTCCGATGACCAGCCGGCCGGCTTTAGTATAAT GGTCAAAGGAGCTATAGTTGTAACAAAGGGATACTGCTAAGAAGGTTGTACGAGGTTT TATAAGTTCACCCAGATTGTTACGATATTTATATATTTATTATATATCTATGTTTAACCTA CAAGAAATAAACCTTTACTAAGGTCTTGTGGTCTGAAATGGCAGATTAGTGCTGTAGAT TTAAGATCTACCCAAAAAGGTTTAAGTCCTTTTTTCAGAATTTAATGTCAACATGATCAC AATTAAGTTTTCAAGATAGAGCTTCCCCATTAATAGAAGAATTAATTATATTCCACGACC ACGCAATATTAGTTTTAACTTTAGTAACAACTTTAGTTGCTTATATTATTTTAACAATATT TAGAAATAAATTTGTAGACCGATTCCTTTTAGAAGGGCATTTAATTGAAGTAATTTGAA CAG
Botryllus schlosseri	TTGTATTTTATTTTTAGAATTTGGTCAAGATTTATTGGTACTGGAATAAG TGTCTTCATTCGTTTAGAATTGTCTCAAGTAGGTCAAGTGGTTAGAGATAGGCAATTGT ATAATGTAATTGTAACTGCTCATGCTTTTGTGATGATTTTCTTTTTTGTTATACCTATGAT AATTAGGAGGTTTGGTAATTGGTTATTACCTTTGATAGTGGGGAGTCCAGATATGGCTT TTCCTCGATTAAATAATATAAGTTTTTGATTGTTGCCCCCTGCTTTGTTTTTTCTTTTTAGA AGTTCTATAATTGAAAGTGGAGTTAGGACTGGGTGAACTGTTTATCCTCCCCTTTCTAG AAATCTAGCTCATTCTAGAGCTGCTTTGGATTGTGCTATTTTTTCTTTACATTTGGCTAG AGTGTCTAGTATTTTAAGATCTCTTAACTTTATGACTACTTTGTTTAATATAAAGGTAAA AGGTTGGGGACTCTTTTCTATATCTTTGTTTTGTTGAACTGTATTGGTCACTACTATTTTG TTATTACTATCTTTACCTGTTTTGGCAGCTGCTATTACTATGTTATTGTTTGATCGAAATT TTAATACTTCTTTTTTTGACCCG

APPENDIX B: PARAMETERS AND PHYLOGENETIC TREES

Table B.1. Parameters for calculation of neighbor joining method

Parameter	Value
Analysis	Phylogeny Reconstruction
statistical method	Neighbor-joining
Test of Phylogeny	Bootstrap method
No. Of Bootstrap replications	1000
Substitution type	Nucleotide
Model	Maximum composite likelihood
Substitutions to include	d: Transitions + Transversions
Rates among sites	Uniform rates
Pattern among Lineages	Homogeneous
Gaps/Missing Data Treatment	Complete deletion
Codons included	1st + 2nd + 3dr + Non Coding

Table B.2. Parameters for calculation of Maximum likelihood method

Parameter	Value
Analysis	Phylogeny Reconstruction
statistical method	Maximum Likelihood
Test of Phylogeny	Bootstrap method
No. Of Bootstrap replications	1000
Substitution type	Nucleotide
Model	Kimura 2-parameter model
ML Heuristic Method	Nearest-Neighbor-Interchange (NNI)
Rates among sites	Uniform rates
Pattern among Lineages	Homogeneous
Gaps/Missing Data Treatment	Complete deletion
Codons included	1st + 2nd + 3dr + Non Coding

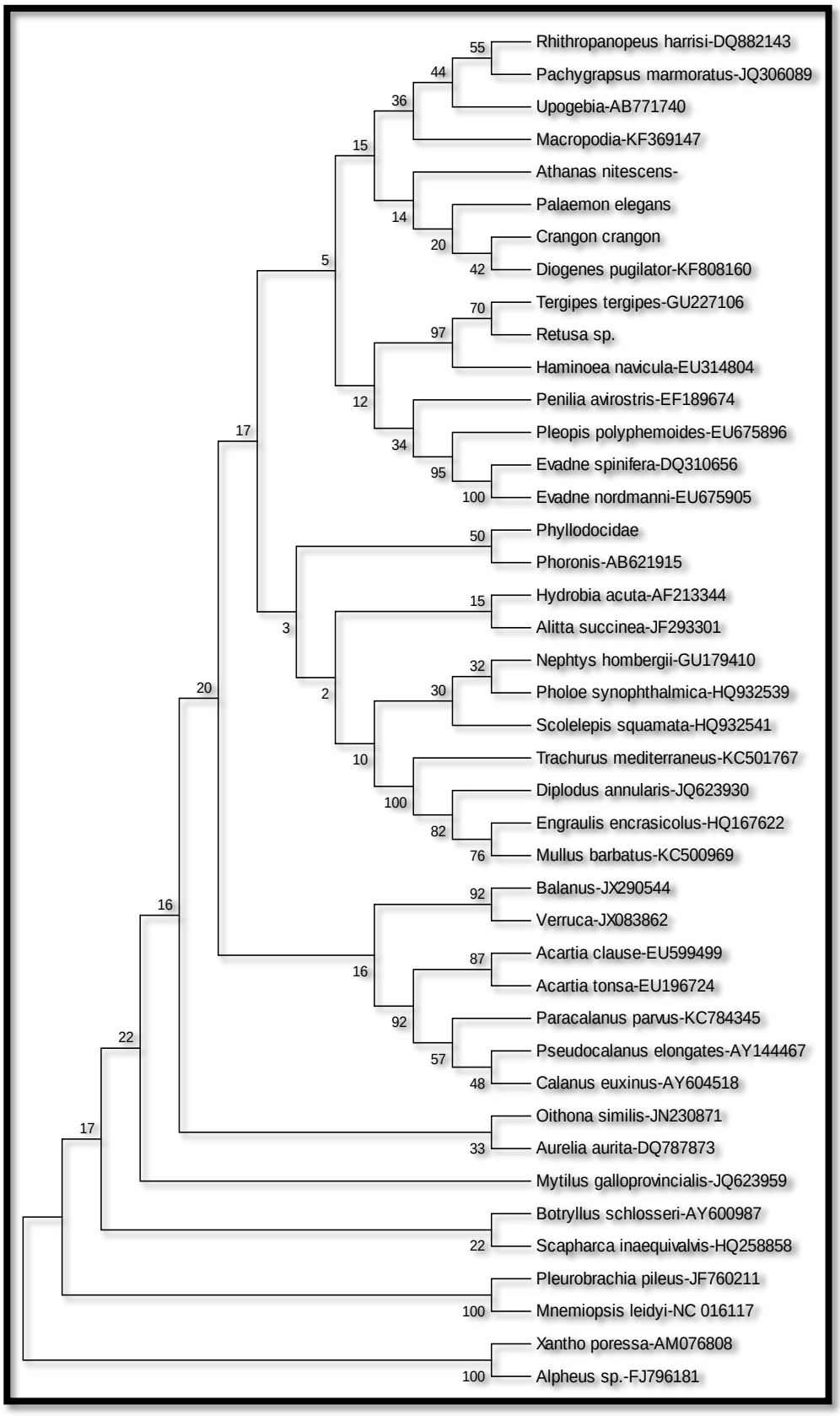


Figure B.1. Phylogenetic tree constructed by Maximum Likelihood method

Table B.3. Parameters for calculation of UPGMA method

Parameter	Value
Analysis	Phylogeny Reconstruction
statistical method	UPGMA
Test of Phylogeny	Bootstrap method
No. Of Bootstrap replications	1000
Substitution type	Nucleotide
Model	Maximum composite likelihood
Substitutions to include	d: Transitions + Transversions
Rates among sites	Uniform rates
Pattern among Lineages	Homogeneous
Gaps/Missing Data Treatment	Complete deletion
Codons included	1st + 2nd + 3dr + Non Coding

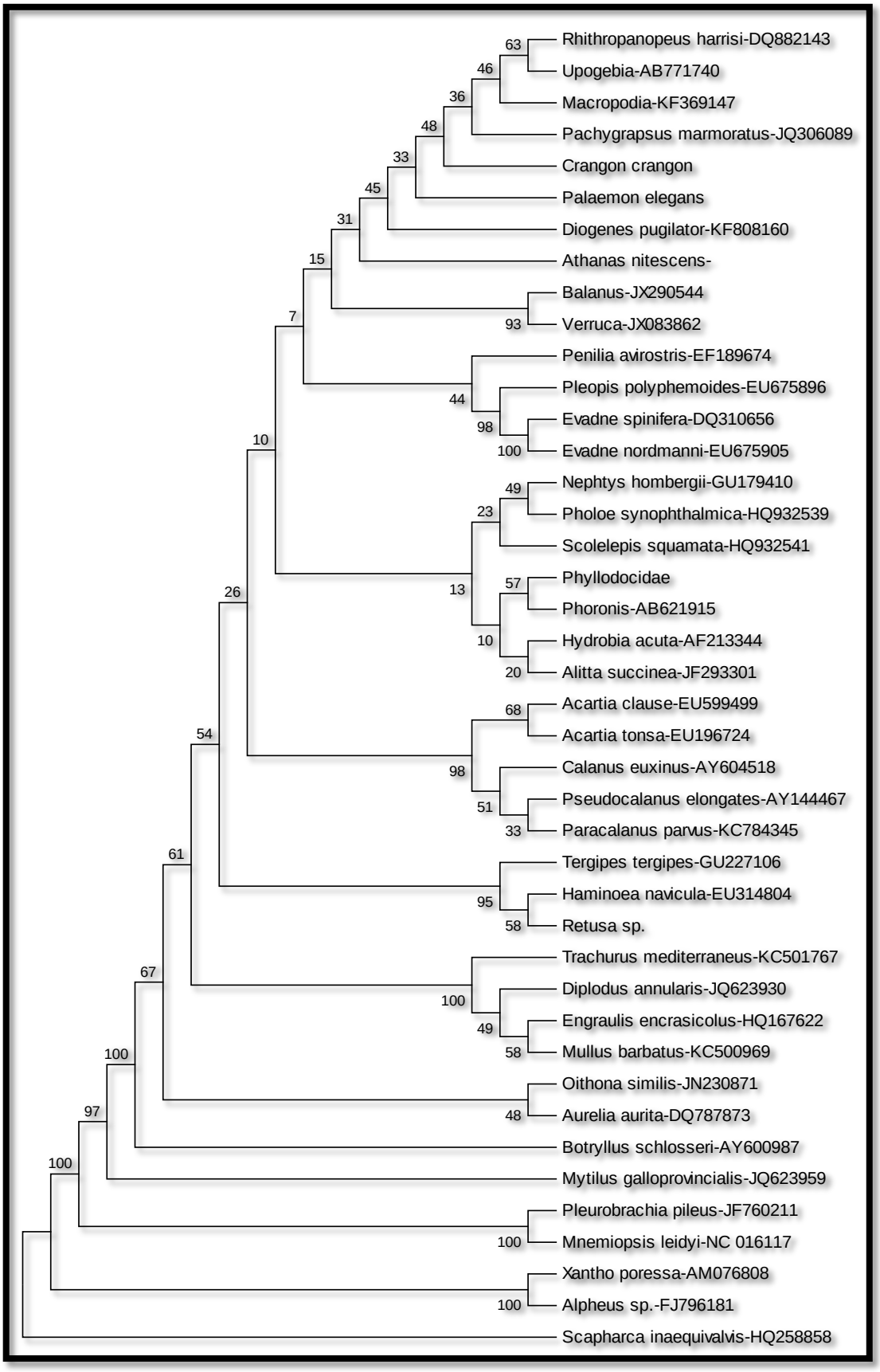


Figure B.2. Phylogenetic tree constructed by UPGMA method

Table B.4. Parameters for calculation of Minimum Evolution method

Parameter	Value
Analysis	Phylogeny Reconstruction
statistical method	Minimum Evolution method
Test of Phylogeny	Bootstrap method
No. Of Bootstrap replications	1000
Substitution type	Nucleotide
Model	Maximum composite likelihood
Substitutions to include	d: Transitions + Transversions
Rates among sites	Uniform rates
Pattern among Lineages	Homogeneous
Gaps/Missing Data Treatment	Complete deletion
Codons included	1st + 2nd + 3dr + Non Coding
ME Heuristic Method	Close-Neighbor-Interchange (CNI)

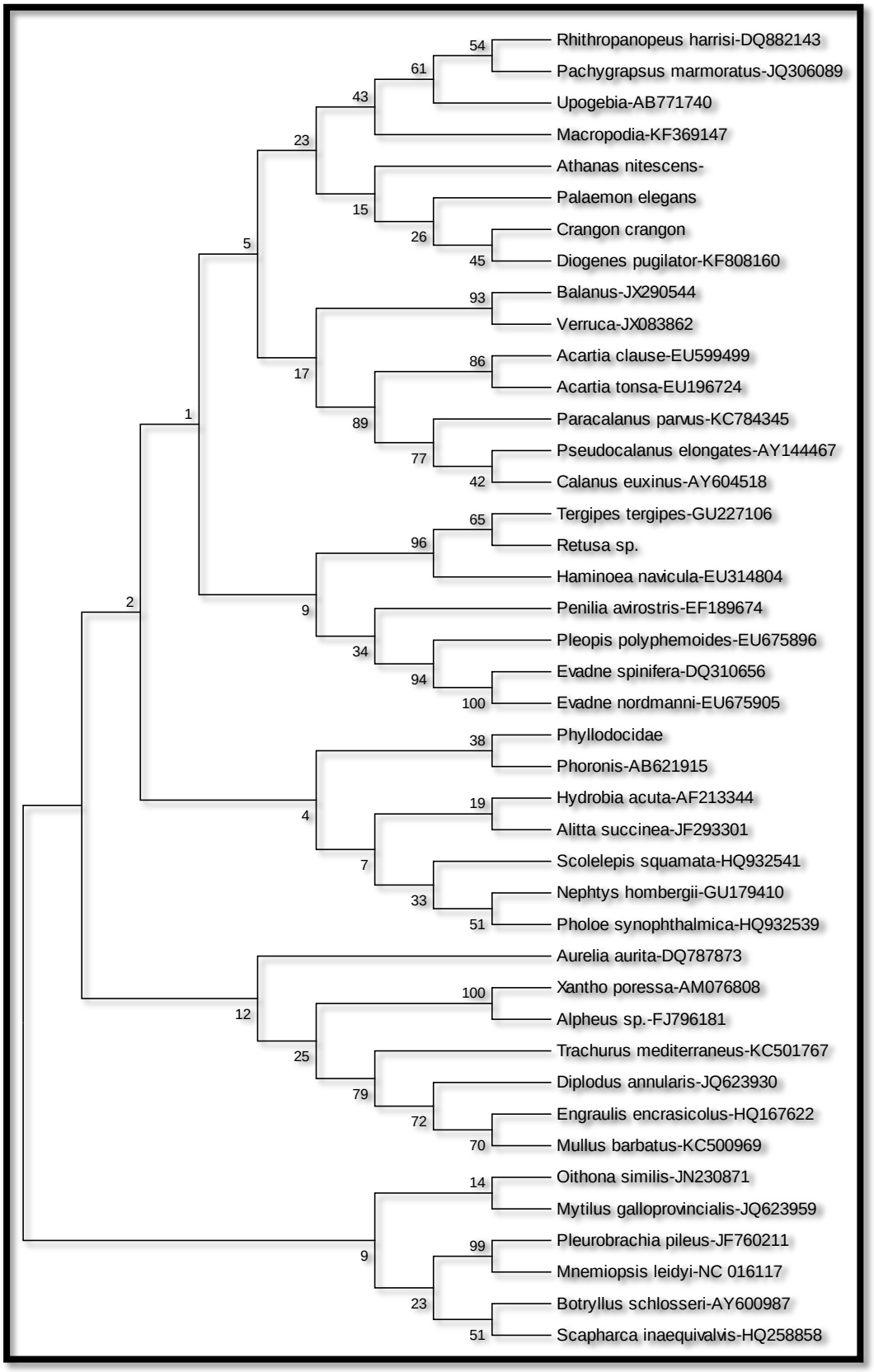


Figure B.3. Phylogenetic tree constructed with minimum evolution method

APPENDIX C: SPECIES SPECIFIC PRIMERS

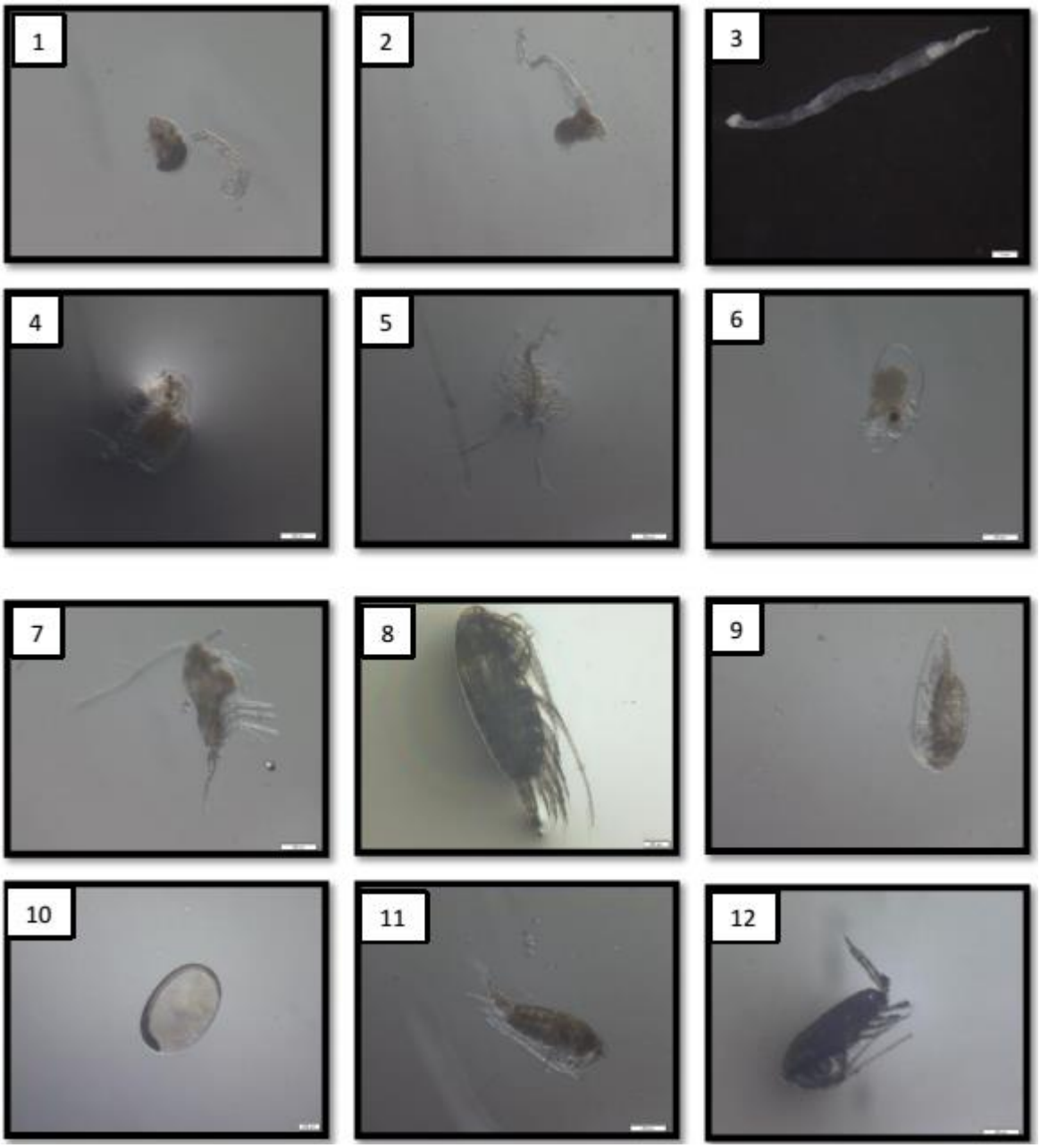
Table C.1. List of Species specific primers

Primer Name	Target Organism	Primer Sequence (5'-3')
acu-forward	*Hydrobia acuta*	ATTGGTGGCTTTGGAAATTG
acu-reverse	*Hydrobia acuta*	TAGGGTCTCCACCTCCTGCT
alp-forward	*Alpheus sp.*	GCCATAGCAGCAATTGGAA
alp-reverse	*Alpheus sp.*	TAAGTCACTGGGGGTTGAGG
ann-forward	*Diplodus annularis*	CCTAAGCCTGCTCATTCGAG
ann-reverse	*Diplodus annularis*	TGGGTCGAAGAAAGTGGTGT
aur-forward	*Aurelia aurita*	GGTGGGAACTGCCTTCAGTA
aur-reverse	*Aurelia aurita*	CTCCAGCAGGGTCAAAGAAG
avi-forward	*Penilia avirostris*	ATTTTGGCTTCTGCCTCCTT
avi-reverse	*Penilia avirostris*	CCCAGGCAAGATCAAAATGT
bar-forward	*Mullus barbatus*	TGCTTGGGCCGGTATAGTAG
bar-reverse	*Mullus barbatus*	GAGCGACAGAAGGAGGAGAA
cla-forward	*Acartia clausii*	GGAGCTTCTGTCGATTTTGC
cla-reverse	*Acartia clausii*	ATTAAACGCACCCCATGAAG
cra-forward	*Crangon crangon*	TTTAATCCTGCCTGCCTTTG
cra-reverse	*Crangon crangon*	CAAACACCGCCCCTATAGAT
ele-forward	*Palaemon elegans*	CGGAGCTTGAGCAGGAATAG
ele-reverse	*Palaemon elegans*	AAAACAGCCCACACGAAAAG
elo-forward	*Pseudocalanus elongatus*	GTTGTCACAGCACACGCTTT
elo-reverse	*Pseudocalanus elongatus*	GCTCCAGCTAAGACGGGTAA
enc-forward	*Engraulis encrasicolus*	CGAGCTGAACTAAGCCAACC
enc-reverse	*Engraulis encrasicolus*	TAATACCAGCGGCAAGGACT
eux-forward	*Calanus euxinus*	TATTTATTGGCCGGTGCGTA
eux-reverse	*Calanus euxinus*	GAGGCATTCGATCAAGCAAT
gal-forward	*Mytilus galloprovincialis*	ACAGTTAGGGCATCCTGGAG

gal-reverse	*Mytilus galloprovincialis*	CCCCTCCTAAAACCGGAATA
har-forward	*Rhithropanopeus harrisii*	AGGTCAACCTGGTACCCTCA
har-reverse	*Rhithropanopeus harrisii*	CTCCAGCAGGATCGAAAAAT
hom-forward	*Nephtys hombergii*	TAGGACAACCCGGTGCTTTA
hom-reverse	*Nephtys hombergii*	CGCAAGAACTGGAAGGGATA
imp-forward	*Balanus improvisus*	GGCCGGCTTTAGTATAATGG
imp-reverse	*Balanus improvisus*	TGCCCTTCTAAAAGGAATCG
ina-forward	*Scapharca inaequivalvis*	TTCCTGAGCGGATACCTGTT
ina-reverse	*Scapharca inaequivalvis*	GAAGTAGCACCCTTCCGTGA
lei-forward	*Mnemiopsis leidyi*	TTCTTTTGTCATGCGTTTAGC
lei-reverse	*Mnemiopsis leidyi*	AACTTCTGGATGGCCAAAAA
mac-forward	*Macropodia sp.*	TATCCGAACCGAACTTGGTC
mac-reverse	*Macropodia sp.*	TAGGATCGCCTCCTCCTCTT
mar-forward	*Pachygrapsus marmoratus*	TTGGAAACTGGCTTGTTCCT
mar-reverse	*Pachygrapsus marmoratus*	CCAGCAGGATCAAAGAATGAA
med-Forward	*Trachurus mediterraneus*	GGGTGCTTGAGCTGGAATAG
med-reverse	*Trachurus mediterraneus*	CCTGCTGGATCAAAGAAAGC
nav-forward	*Haminoea navicula*	CGGTTCGAACTAGGAACAGC
nav-reverse	*Haminoea navicula*	CCGGATCAAAGAACCTCGTA
nit-forward	*Athanas nitescens*	TACCGCCCATGCCTTTATTA
nit-reverse	*Athanas nitescens*	TGTGTTGAGGTTTCGGTCTG
nor-forward	*Evadne nordmanni*	TAGGACAGGCAGGGAGCTTA
nor-reverse	*Evadne nordmanni*	CCGGTCCGTTAGAAGCATAG
par-forward	*Paracalanus parvus*	GGTTCTTTAATTGGCGACGA
par-reverse	*Paracalanus parvus*	ACCACCCCCAACATCATAAA
pho-forward	*Pholoe sp.*	GTGACAGCACATGCGTTTCT
pho-reverse	*Pholoe sp.*	GGCGGTTACTTTAGCAGCTC
phs-forward	*Phoronis sp.*	TTTTCGGAGTGTGAACAGGA
phs-reverse	*Phoronis sp.*	CGGAACACGCTCTAATTGGT
phy-forward	*Phyllodoce sp.(groenlandica)*	TGATCTGGGCTTCTTGGAAC
phy-reverse	*Phyllodoce*	AAAGGAACCCGCTCTAATCG

	sp.(groenlandica)	
pil-forward	*Pleurobrachia pileus*	GGTTGGACGTTATACCCACCT
pil-reverse	*Pleurobrachia pileus*	GATGAGCCCAAACAACACAA
pol-forward	*Pleopis polyphemoides*	TGATATGGCTTTCCCTCGAC
pol-reverse	*Pleopis polyphemoides*	CCCAGCTGGATCAAAGAATG
por-forward	*Xantho poressa*	ATTCTTCCCGCCTTTGGTAT
por-reverse	*Xantho poressa*	GAATCAATGGGCAATTCCAG
pug-forward	*diogenes pugilator*	GGTCAACAAATCATAAAGAYA TYGG
pug-reverse	*diogenes pugilator*	TAAACTTCAGGGTGACCAAAR AAYCA
ret-forward	*Retusa sp*	CCTTTCTTGGTGACGACCAT
ret-reverse	*Retusa sp*	CCGGATCGAAAAATCTGGTA
sch-forward	*Botryllus schlosseri*	CTGCTCATGCTTTTGTGATGA
sch-reverse	*Botryllus schlosseri*	ATAGCAGCTGCCAAAACAGG
sim-forward	*Oithona similis*	CTGGAGTTTGGGCAGGAATA
sim-reverse	*Oithona similis*	TCGTAATTGCCCCAGCTAAC
spi-forward	**Evadne spinifera*	TAGGACAGGCAGGGAGCTTA
spi-reverse	**Evadne spinifera*	GCAGGATCGAAGAACGATGT
sq-forward	*Scolelepis squamata*	TTATTCGGGCCGAGTTAGGT
squ-reverse	*Scolelepis squamata*	TAGACGAAGGCCATCTCACC
su-forward	*Alitta succinea*	GCCCTACTTGGAAGAGACCA
suc-reverse	*Alitta succinea*	GGGGTACTCGTTCTAAGCTAA
ter-forward	*Tergipes tergipes*	GGGGTGGTGACCCTATTCTT
ter-reverse	*Tergipes tergipes*	AGAACACCACGCTGTGACTG
ton-forward	*Acartia tonsa*	GCTGGAAGGCTAATTGGAGA
ton-reverse	*Acartia tonsa*	TGCTCACGCAAACAAAGGTA
upo-forward	*Upogebia*	AGCCGGAATAGTGGGAACTT
upo-reverse	*Upogebia*	CTCCTCCAGCTGGGTCAA
ver-forward	*Verruca sp.*	GCTGAATTAGGTCAACCAGGA
ver-reverse	*Verruca sp.*	AGCGCCTGCTAAAACAGGTA

APPENDIX D: PHOTOGRAPHS OF THE ZOOPLANKTON SAMPLES



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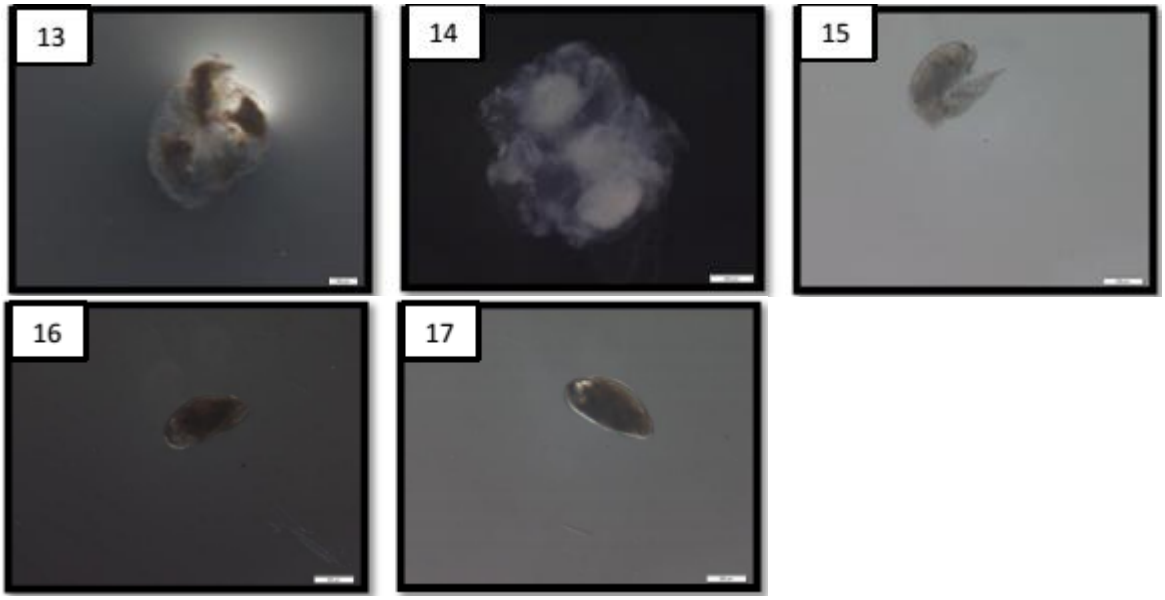
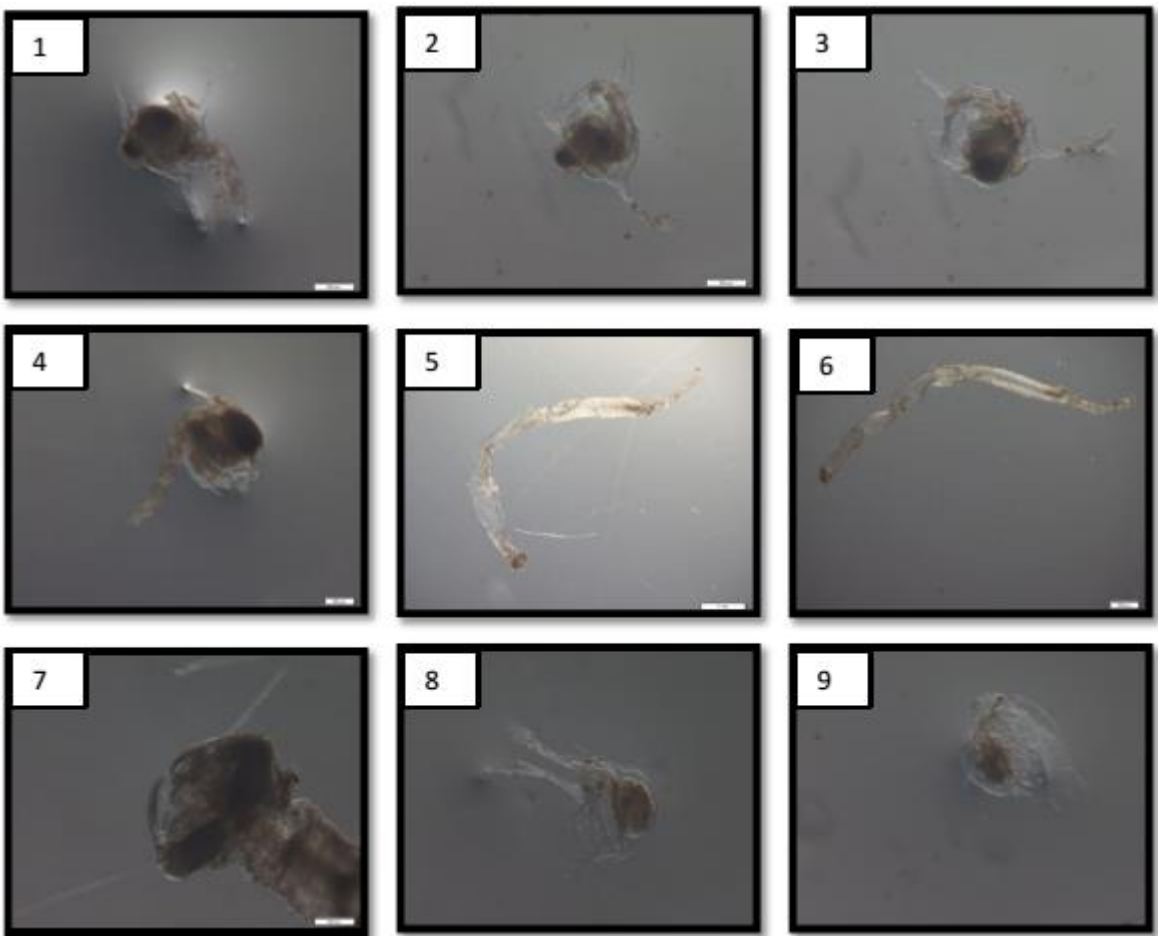


Figure D.1. Samples from 1. Station;

1-Oikopleura dioica, 2-Oikopleura dioica, 3-Sagitta setosa, 4-Penilia avirostris, 5-Penilia avirostris, 6-Evadne spinifera, 7-Acartia sp., 8-Calanus euxinus, 9-Centropages, ponticus, 10-Centropages ponticus, 11-Pseudocalanus elongates, 12-Ctenophora (cydipid) sp1, 13-Engraulis encrasicolus egg, 14-Hydromedusae, 15-Ostracoda, 16-Ostracoda, 17-Ostracoda (identifications were performed by İlayda Destan Öztürk and Dr. Yeşim Ak Örek)



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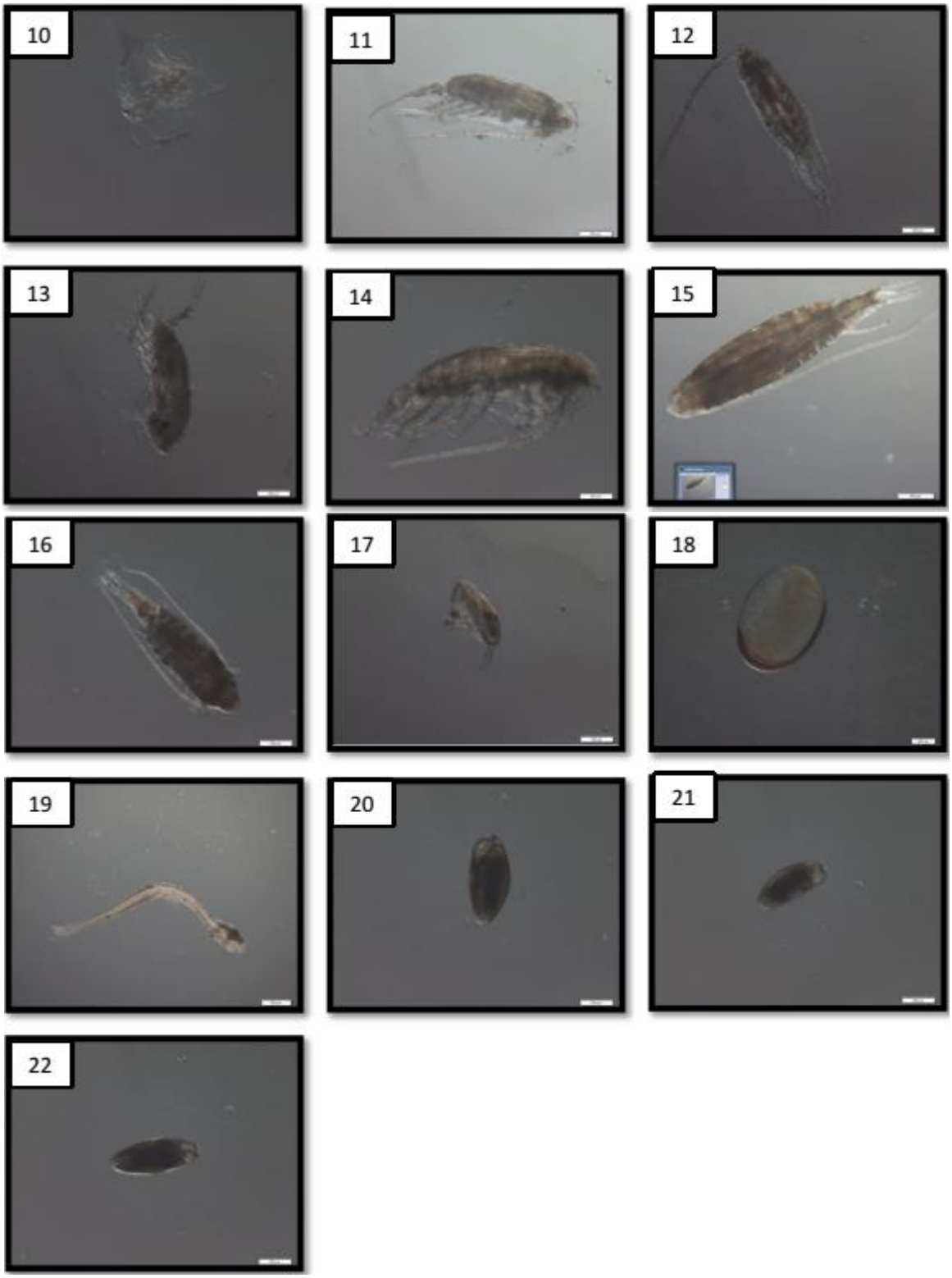

Figure D.2. Samples from 2. Station

1-Brachyura sp, 2-Brachyura sp1, 3-Brachyura sp2, 4-Brachyura sp3, 5-Sagitta setosa, 6-Sagitta setosa, 7-Sagitta setosa, 8-Penilia avirostris, 9-Penilia avirostris, 10-Penilia avirostris, 11-Acartia sp., 12-Acartia sp., 13-Acartia sp., 14-Calanus euxinus, 15-Calanus euxinus, 16-Centropages ponticus, 17-Paracalanus parvus, 18-Engraulis encrasicolus egg, 19-Engraulis encrasicolus larvae, 20-Ostracoda, 21-Ostracoda, 22-Ostracoda2 (identifications were performed by İlayda Destan Öztürk and Dr. Yeşim Ak Örek)

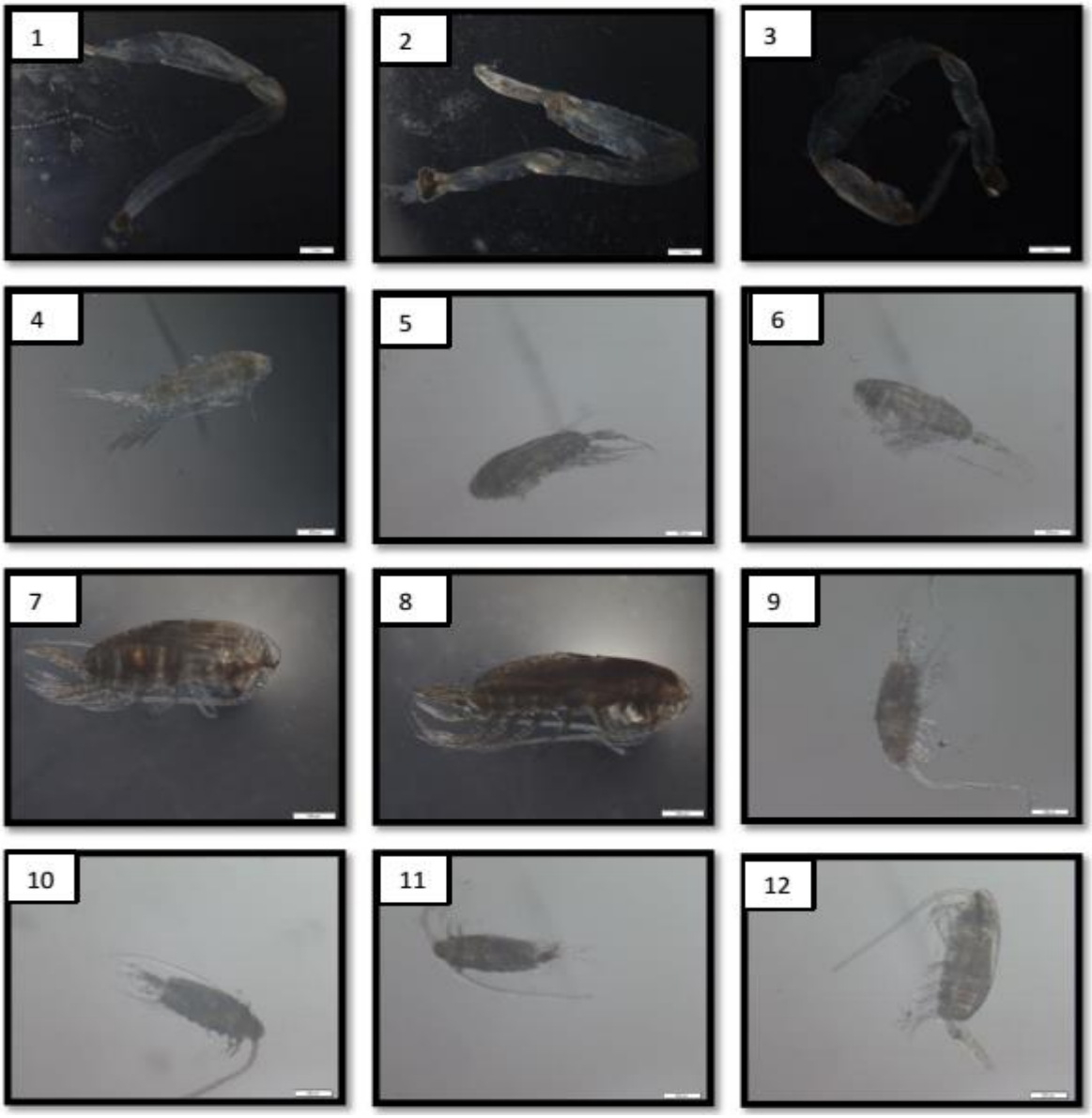

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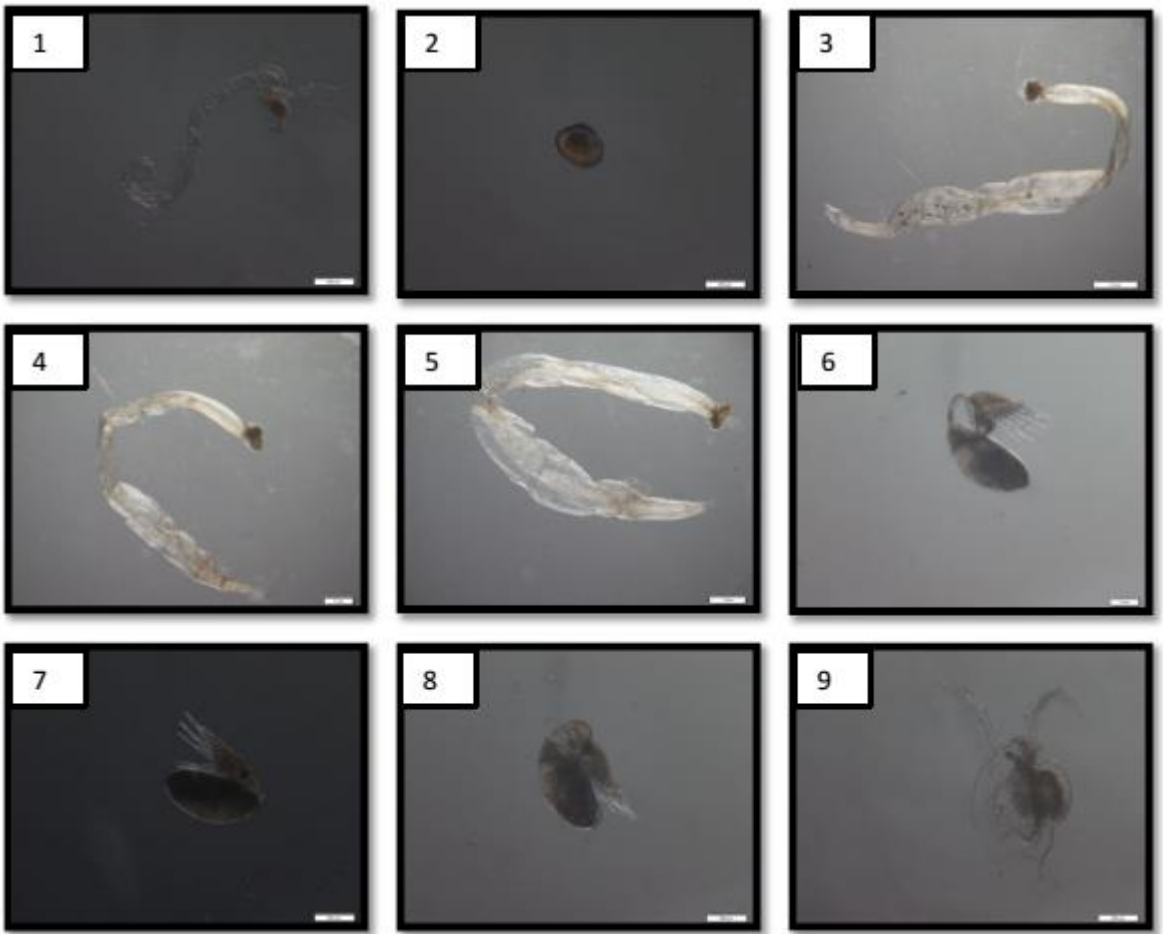
Figure D.3. Samples from 3. Station

1-Sagitta setosa, 2-Sagitta setosa, 3-Sagitta setosa, 4-Ostracoda, 5-Ostracoda, 6-Acartia sp., 7-Acartia sp., 8-Acartia sp., 9-Calanus euxinus, 10-Calanus euxinus, 11-Centropages ponticus, 12-Centropages ponticus, 13-Centropages ponticus, 14-Pseudocalanus elongates, 15-Pseudocalanus elongates, 16-Engraulis encrasicolus larvae (identifications were performed by İlayda Destan Öztürk and Dr. Yeşim Ak Örek)



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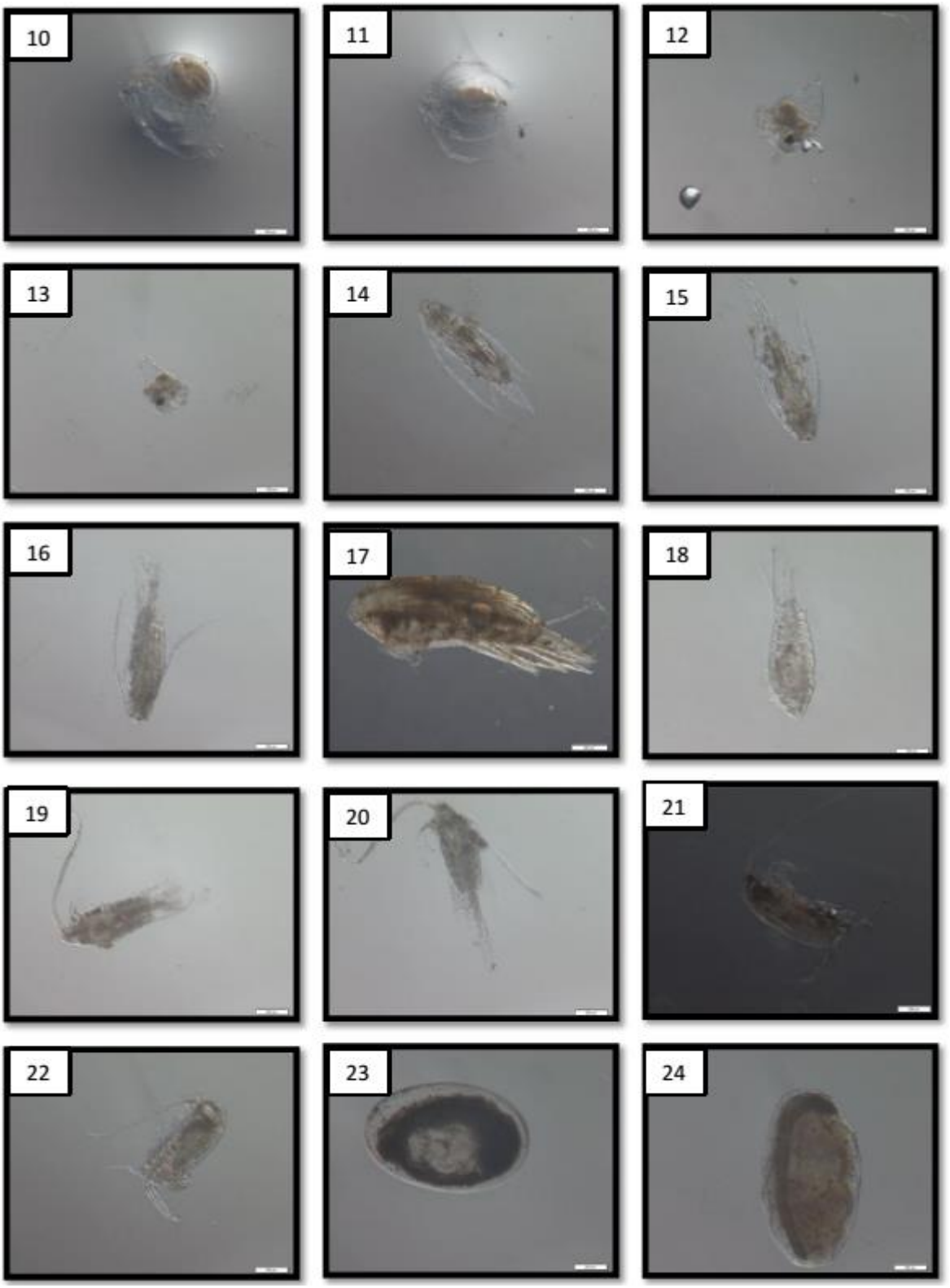

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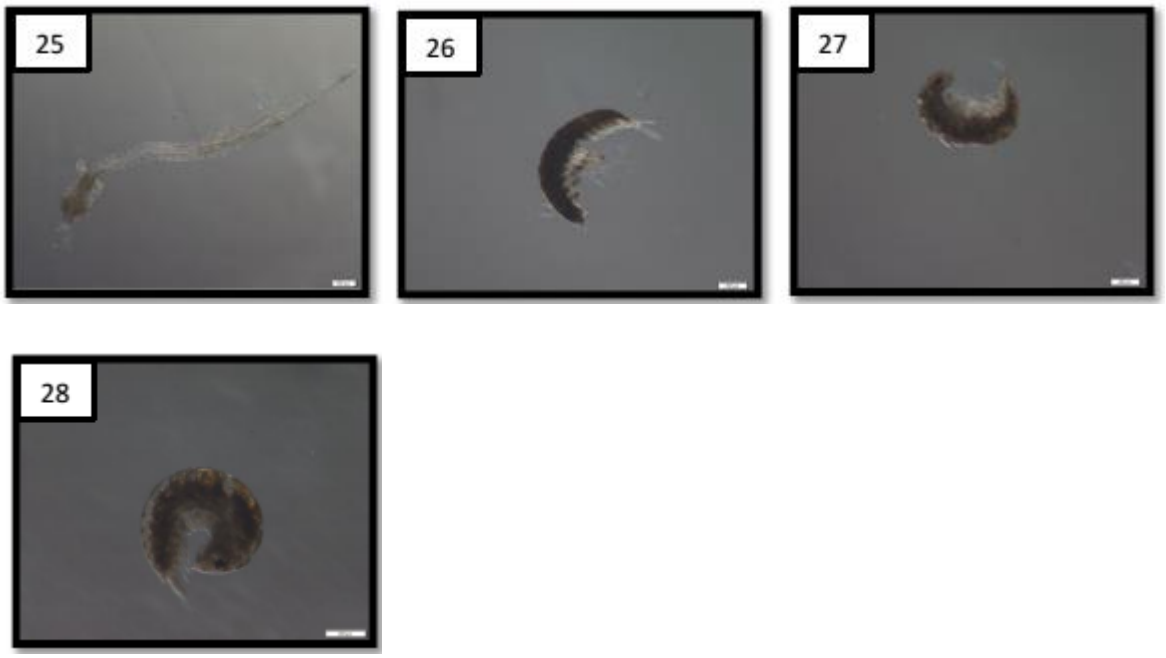
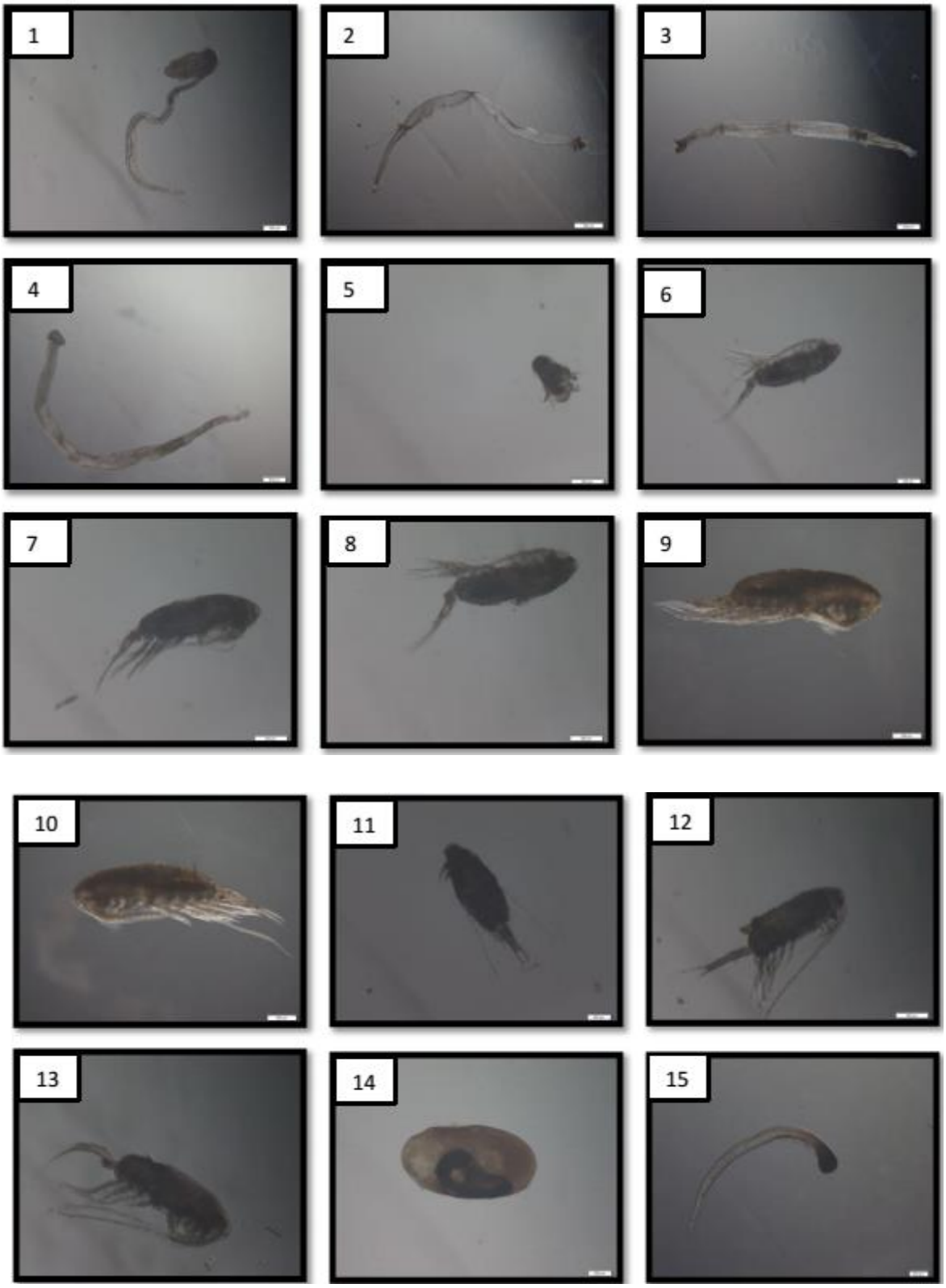


Figure D.4. Samples from 4. Station

1-Oikopleura dioica, 2-Bivalvia, 3-Sagitta setosa, 4-Sagitta setosa, 5-Sagitta setosa, 6-Cirripedia Larvae, 7-Cirripedia Larvae, 8-Cirripedia Larvae, 9-Penilia avirostris 10-Penilia avirostris, 11-Penilia avirostris, 12-Evadne spinifera, 13-Evadne spinifera, 14-Acartia sp., 15-Acartia sp., 16-Acartia sp., 17-Calanus euxinus, 18-Centropages ponticus, 19-Centropages ponticus, 20-Centropages ponticus, 21-Pseudocalanus elongates, 22-Pseudocalanus elongates, 23-Engraulis encrasicolus egg, 24-Engraulis encrasicolus egg, 25-Engraulis encrasicolus larvae, 26-Isopoda sp1, 27-Isopoda sp1, 28-Isopoda larvae (identifications were performed by İlayda Destan Öztürk and Dr. Yeşim Ak Örek)



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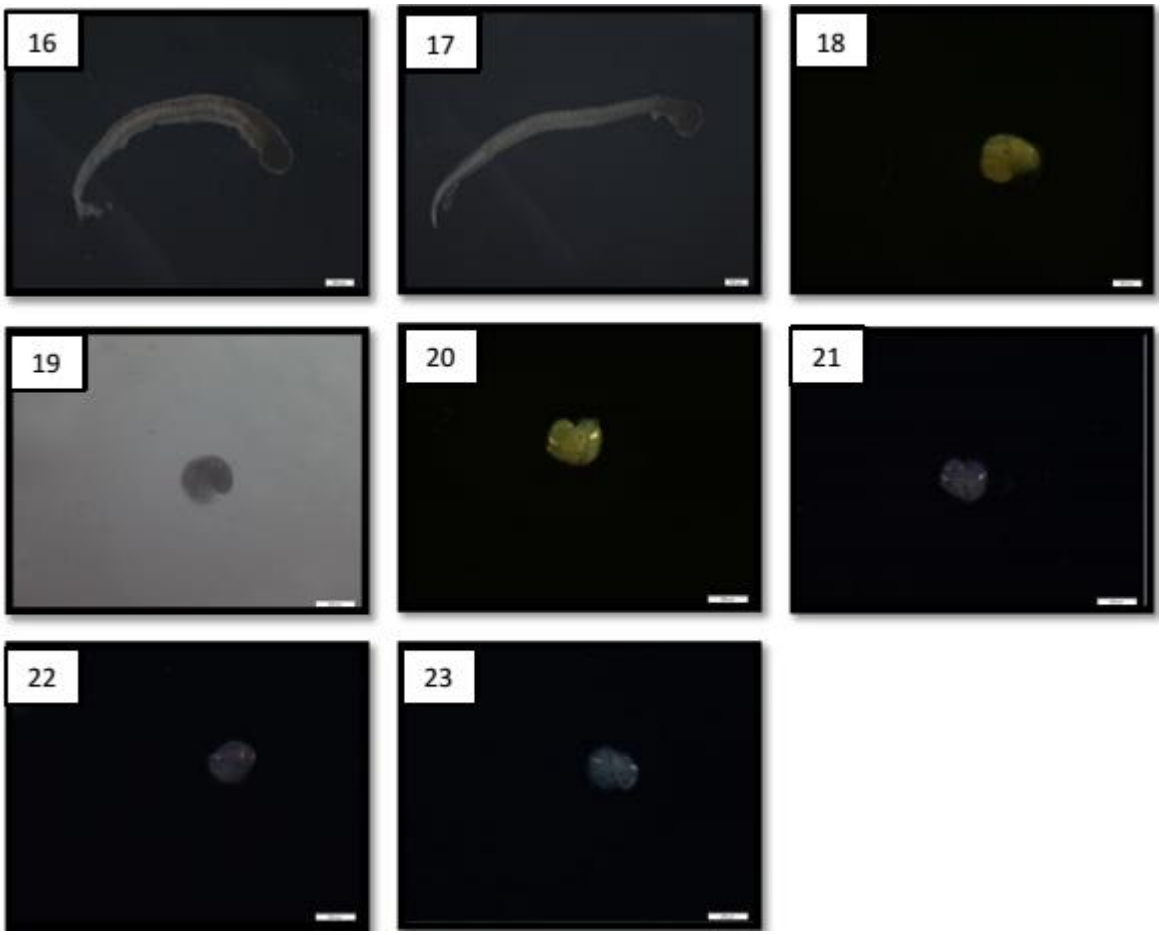
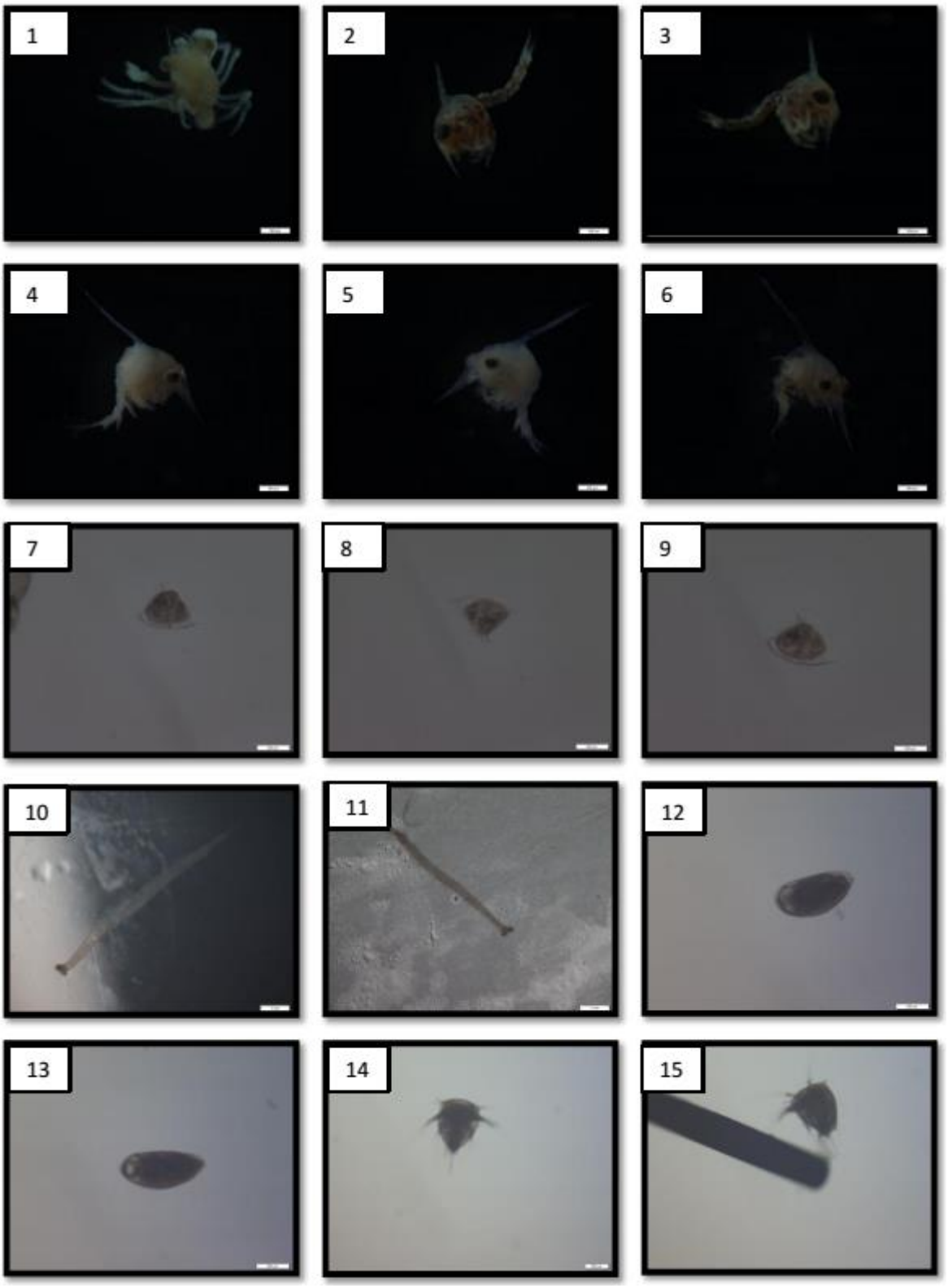


Figure D.5. Samples from 5. Station

1-Oikopleura dioica, 2-Sagitta setosa, 3-Sagitta setosa, 4-Sagitta setosa, 5-Podon sp., 6-Acartia sp., 7-Acartia sp., 8-Acartia sp., 9-Calanus euxinus, 10-Calanus euxinus, 11-Centropages ponticus, 12-Pseudocalanus elongates, 13-Pseudocalanus elongates, 14-Engraulis encrasicolus egg, 15-Engraulis encrasicolus larvae, 16-Engraulis encrasicolus larvae, 17-Engraulis encrasicolus larvae, 18-Gastropoda_sp1, 19-Gastropoda_sp4, 20-Gastropoda_sp4, 21-Gastropoda_sp4, 22-Gastropoda_sp4, 23-Gastropoda_sp4 (identifications were performed by İlayda Destan Öztürk and Dr. Yeşim Ak Örek)



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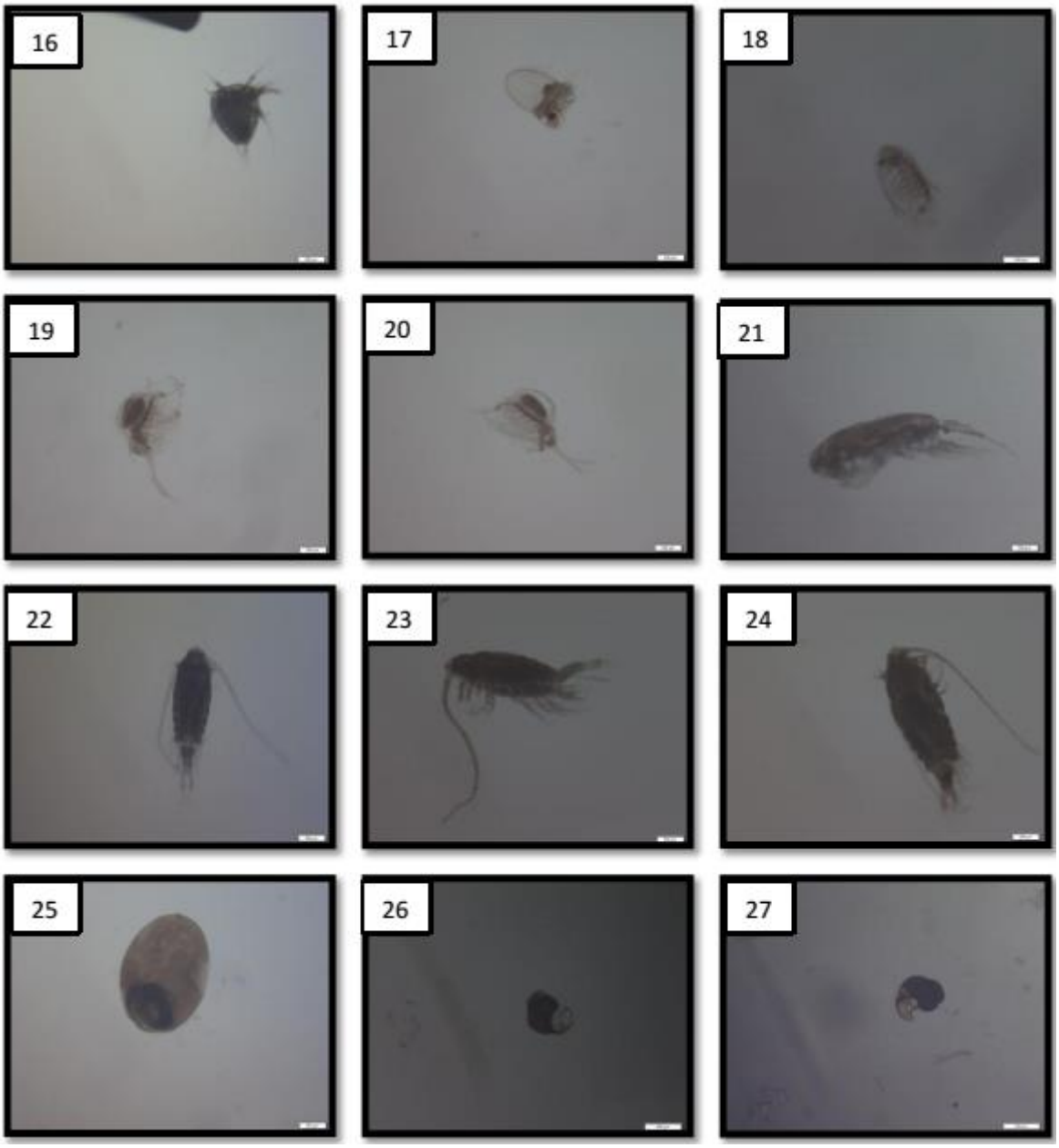

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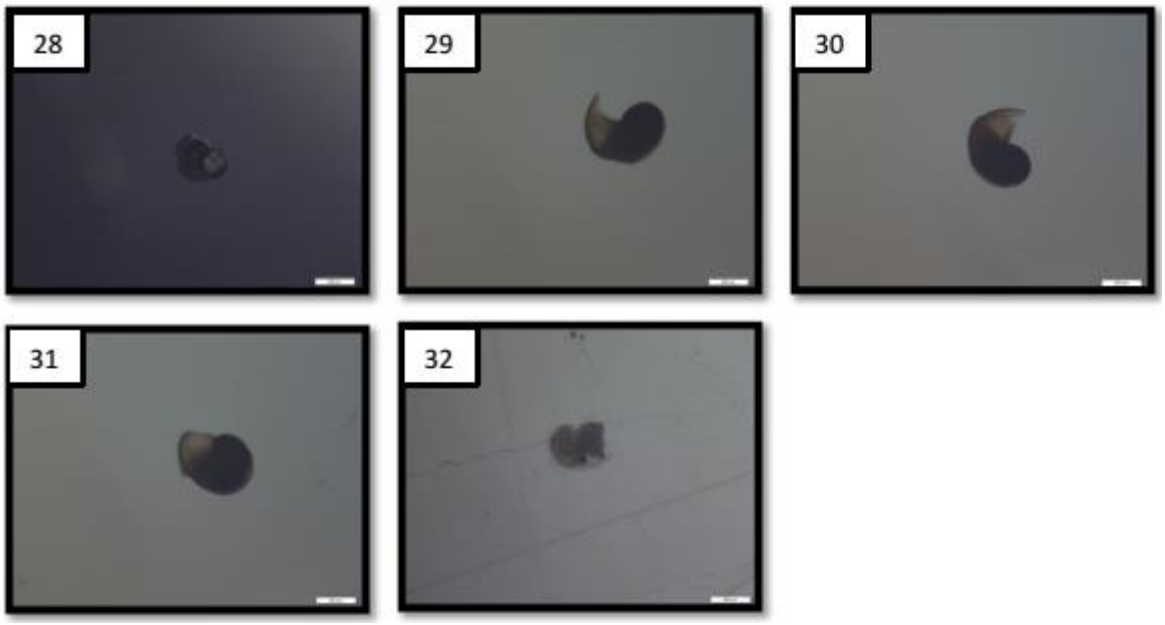
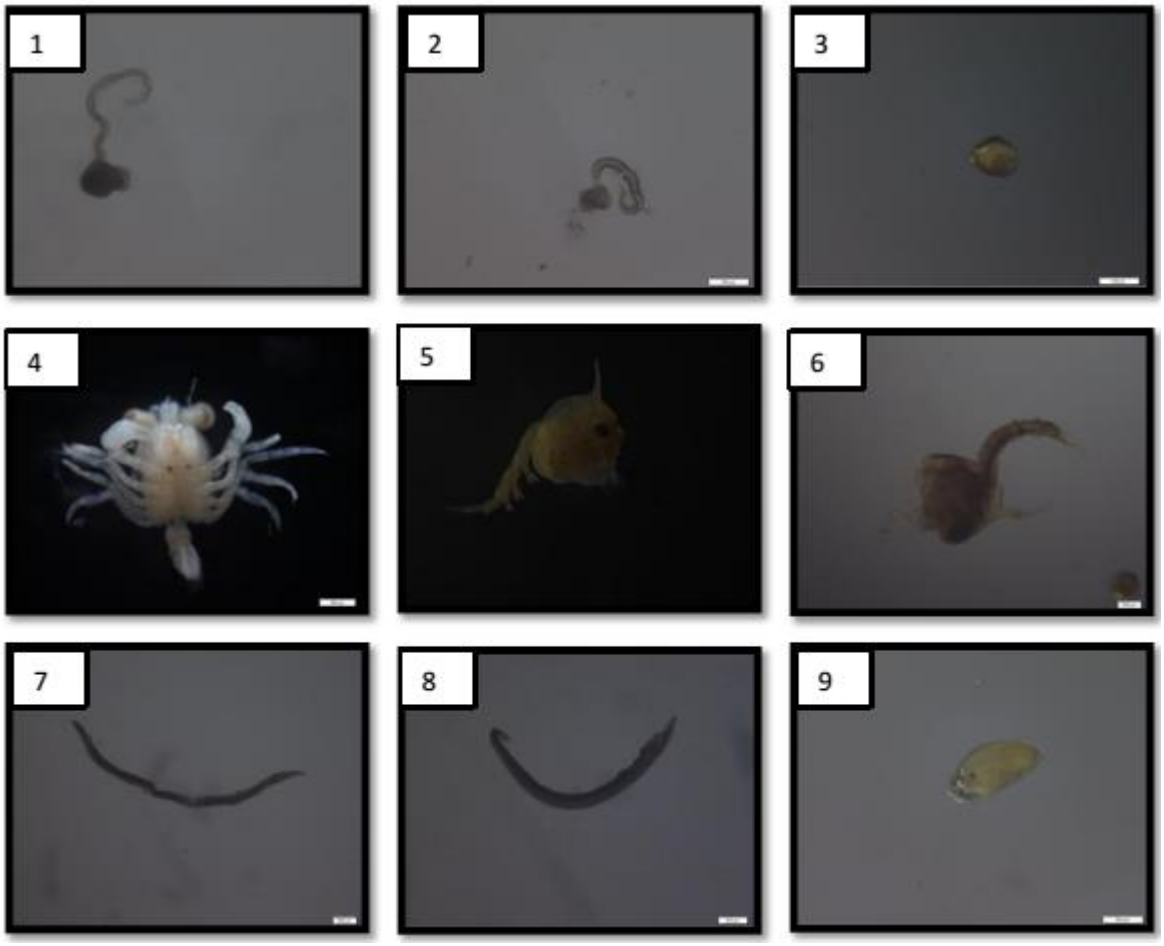


Figure D.6. Samples from 6. Station

1-Brachyura Megalopa Larvae sp1, 2-Brachyura sp6, 3-Brachyura sp6, 4-Brachyura sp7, 5-Brachyura sp7, 6-Brachyura sp7, 7-Ctenophora (cydipid) sp1, 8-Ctenophora (cydipid) sp1, 9-Ctenophora (cydipid) sp1, 10-Sagitta setosa, 11-Sagitta setosa, 12-Cirripedia Larvae, 13-Cirripedia Larvae, 14-Cirripedia Larvae sp2, 15-Cirripedia Larvae sp2, 16-Cirripedia Larvae sp2, 17-Evadne tergestina, 18-Penilia avirostris, 19-Penilia avirostris, 20-Penilia avirostris, 21-Acartia sp., 22-Centropages ponticus, 23-Centropages ponticus, 24-Centropages ponticus, 25-Engraulis encrasicolus egg, 26-Gastropoda_sp1, 27-Gastropoda_sp1, 28-Gastropoda_sp1, 29-Gastropoda_sp2, 30-Gastropoda_sp2, 31-Gastropoda_sp2, 32-Gastropoda_sp3(identifications were performed by İlayda Destan Öztürk and Dr. Yeşim Ak Örek)



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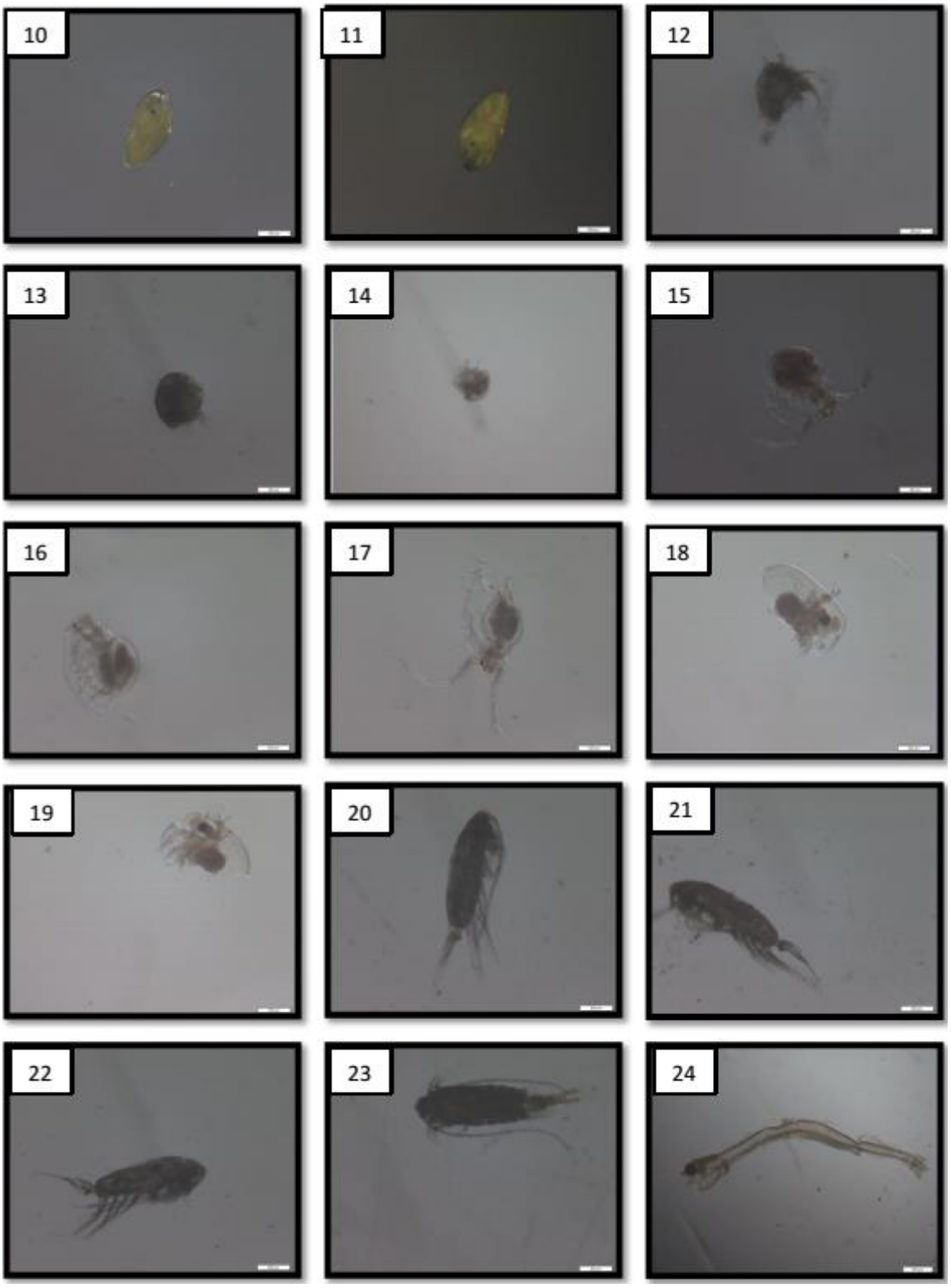

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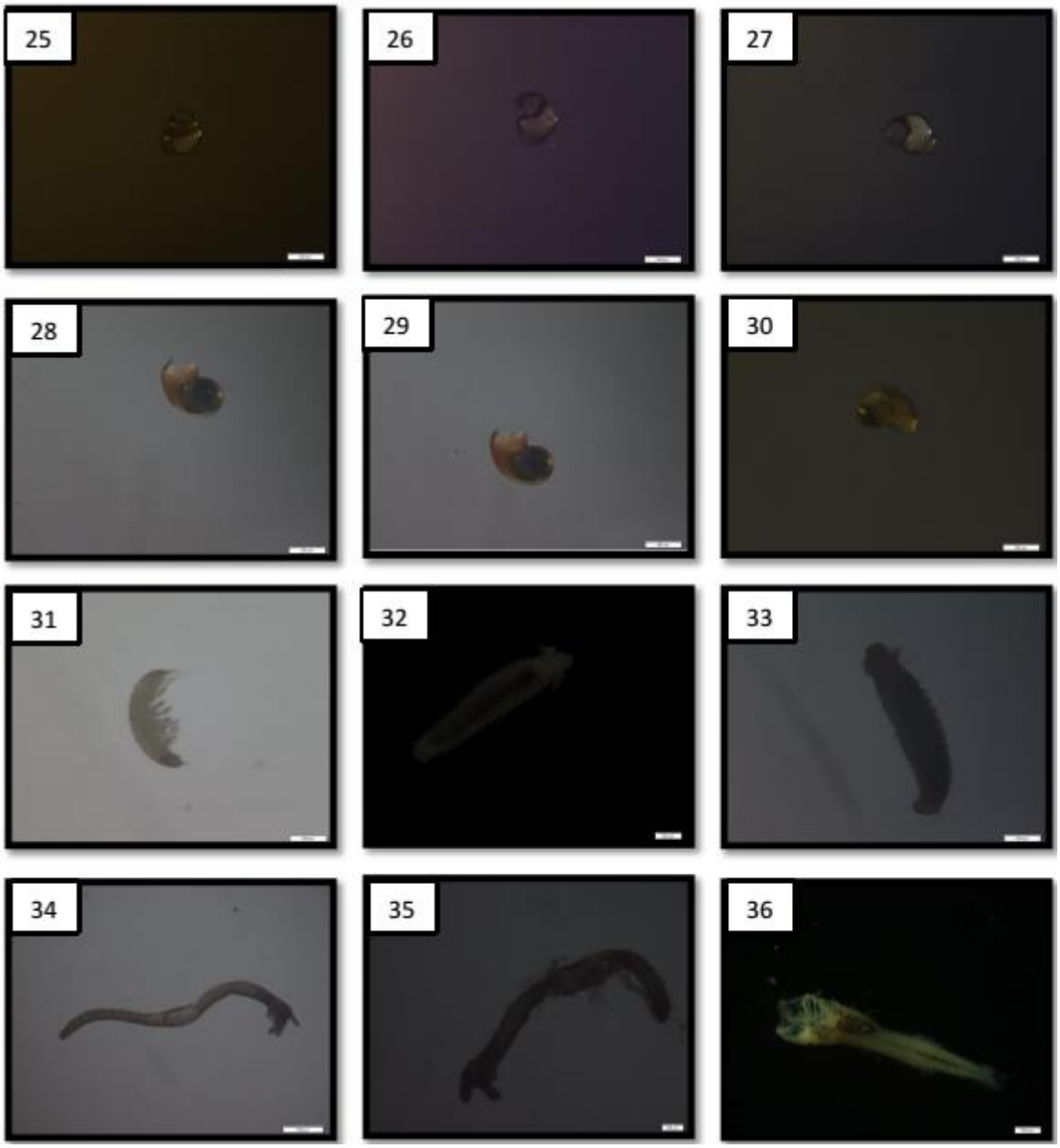

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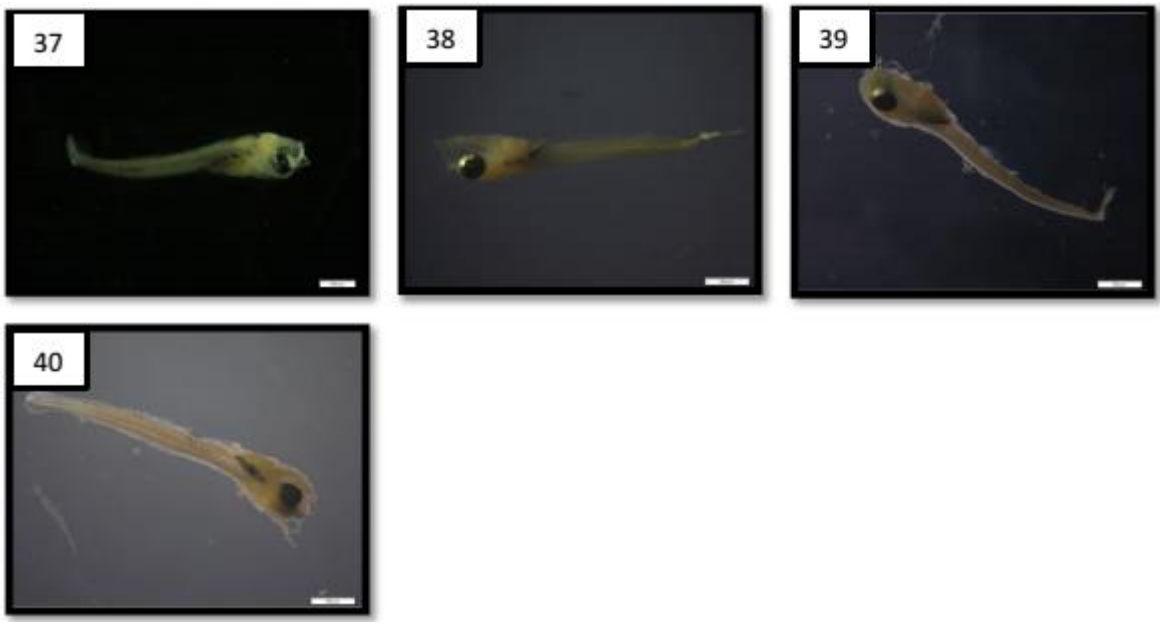

Figure D.7. Samples from 7. Station

1-Oikopleura dioica, 2-Oikopleura dioica, 3-Bivalvia, 4-Brachyura Megalopa Larvae sp2, 5-Brachyura sp4, 6-Brachyura sp5, 7-Cephalochordata, 8-Cephalochordata, 9-Cirripedia Larvae sp1, 10-Cirripedia Larvae sp1, 11-Cirripedia Larvae sp1, 12-Cirripedia Larvae sp3, 13-Cirripedia Larvae sp3, 14-Cirripedia Larvae sp2, 15-Penilia avirostris, 16-Penilia avirostris, 17-Penilia avirostris, 18-Evadne spinifera, 19-Evadne spinifera, 20-Acartia sp., 21-Acartia sp., 22-Acartia sp., 23-Centropages ponticus, 24-Engraulis encrasicolus larvae, 25-Gastropoda_sp1, 26-Gastropoda_sp1, 27-Gastropoda_sp1, 28-Gastropoda_sp2, 29-Gastropoda_sp2, 30-Gastropoda_sp2, 31-Isopoda larvae, 32-Polychaeta, 33-Polychaeta, 34-Polychaeta, 35-Polychaeta, 36-Polychaeta, 37-Teleostei Larvae Mullus barbatus, 38-Teleostei Larvae Mullus barbatus, 39-Teleostei Larvae sp1, 40-Teleostei Larvae sp1, 41-Teleostei Larvae sp1(identifications were performed by İlayda Destan Öztürk and Dr. Yeşim Ak Örek)

APPENDIX E: VISUAL OUTPUTS OF QPCR

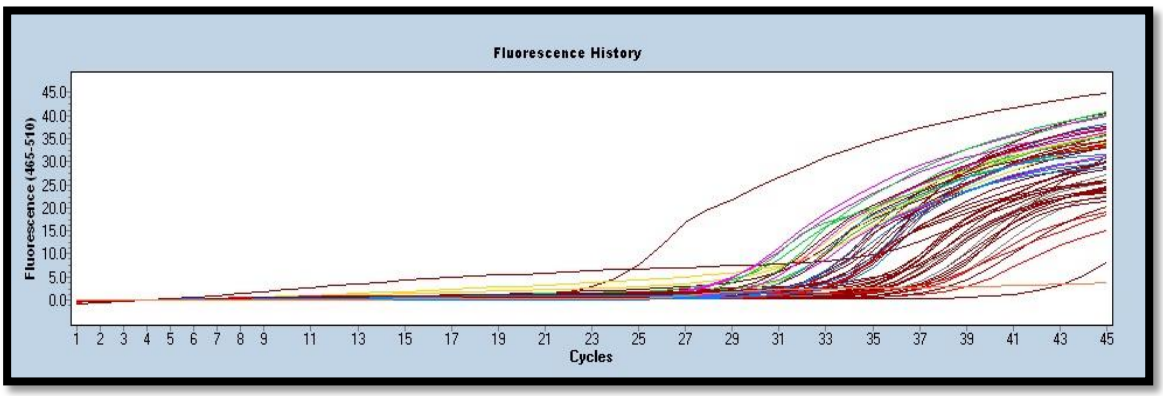

Figure E.1. Amplification curve plot of fish species obtained from Roche LightCycler 480 device

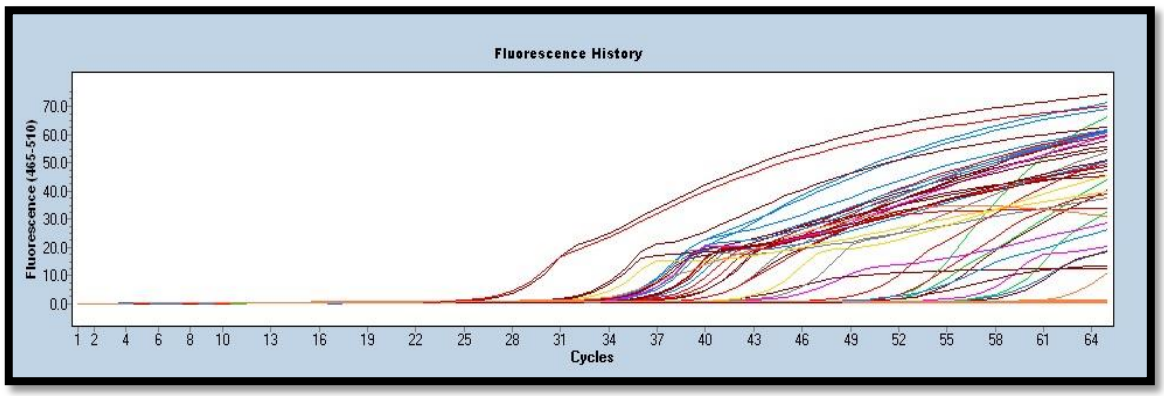

Figure E.2. Zooplankton amplification curve plot of 1. station obtained from Roche LightCycler 480 device

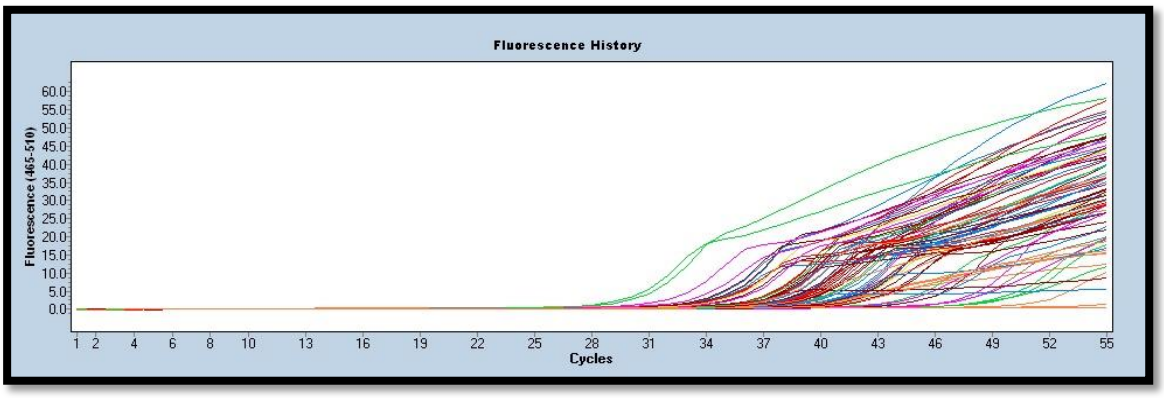

Figure E.3. Zooplankton amplification curve plot of 2. station obtained from Roche LightCycler 480 device

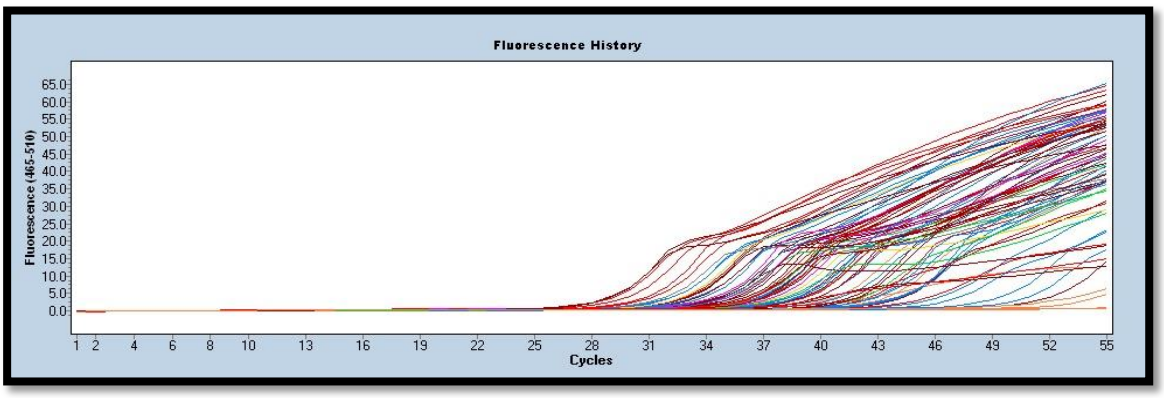

Figure E.4. Zooplankton amplification curve plot of 3. station obtained from Roche LightCycler 480 device

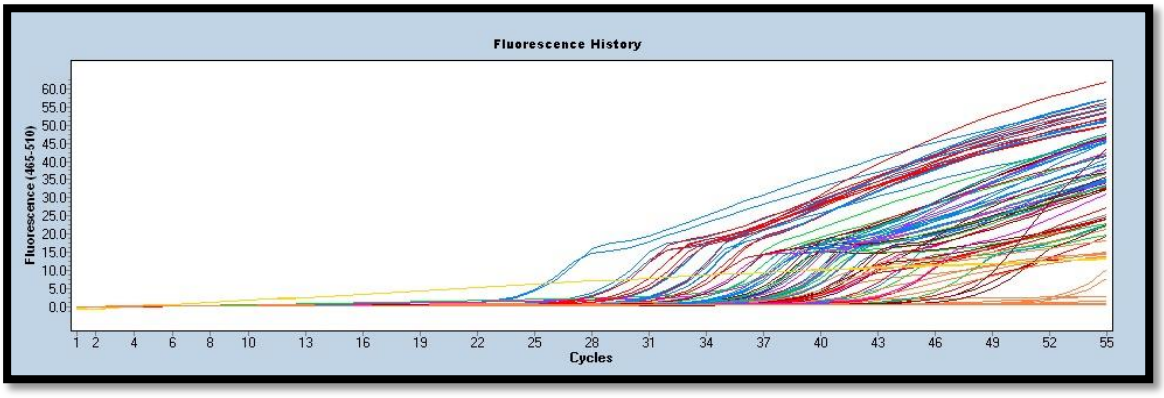

Figure E.5. Zooplankton amplification curve plot of 4. station obtained from Roche LightCycler 480 device

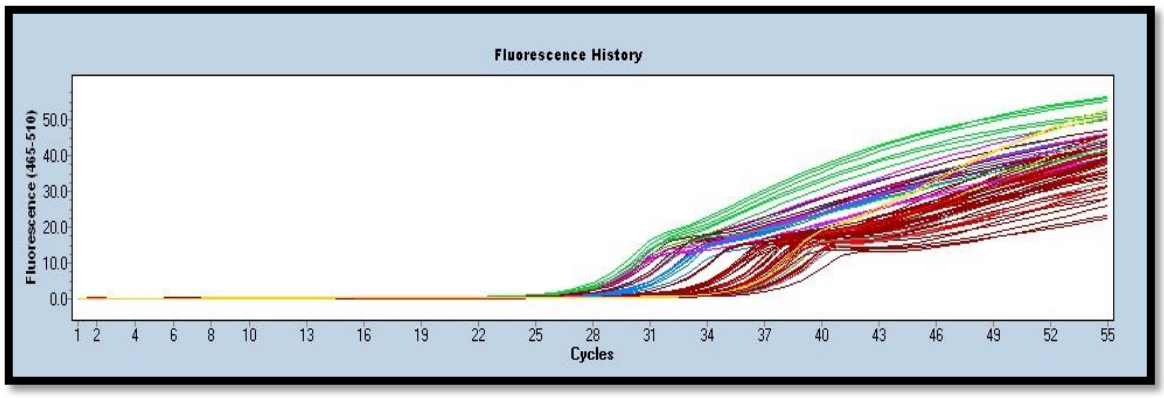

Figure E.6. Zooplankton amplification curve plot of 5. station obtained from Roche LightCycler 480 device

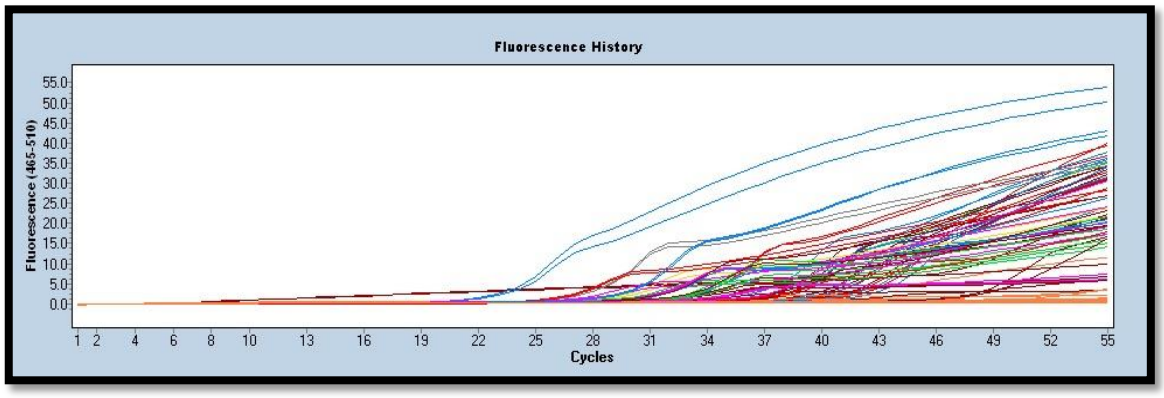

Figure E.7. Zooplankton amplification curve plot of 5. station obtained from Roche LightCycler 480 device

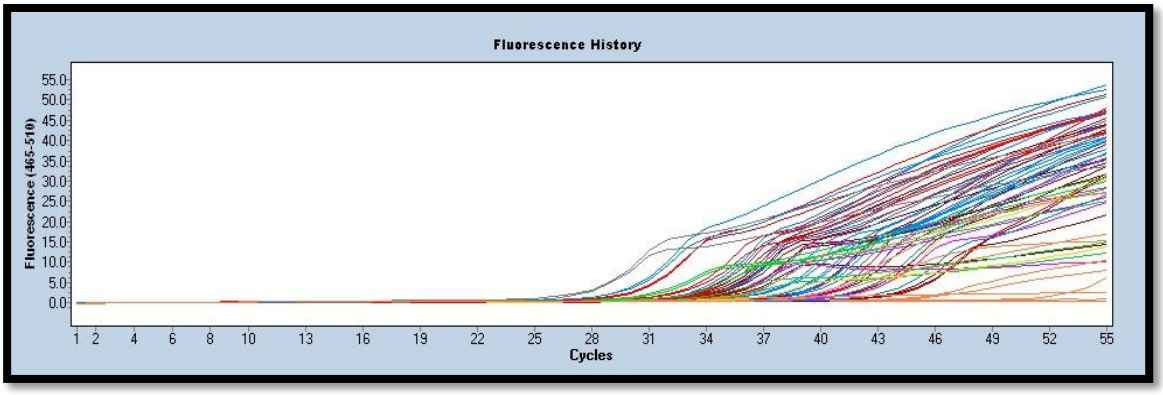

Figure E.8. Zooplankton amplification curve plot of 6. station obtained from Roche LightCycler 480 device

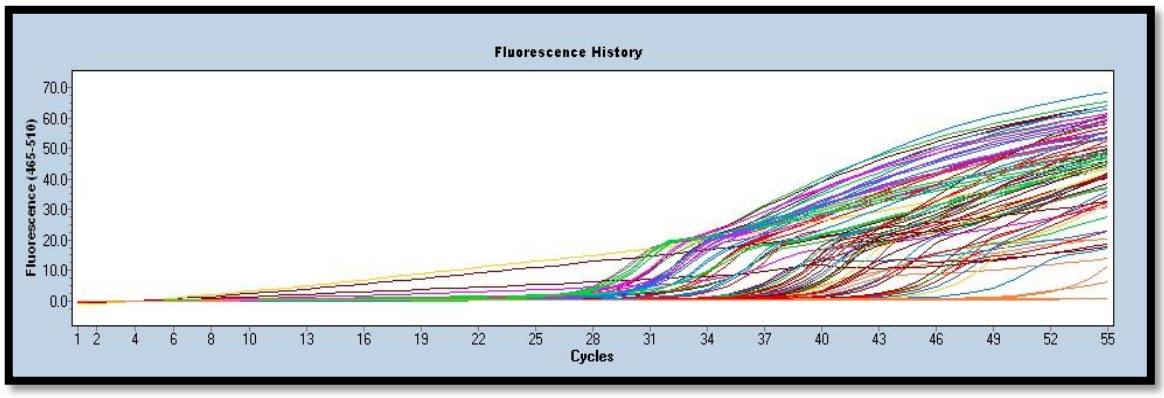

Figure E.9. Zooplankton amplification curve plot of 6. station obtained from Roche LightCycler 480 device

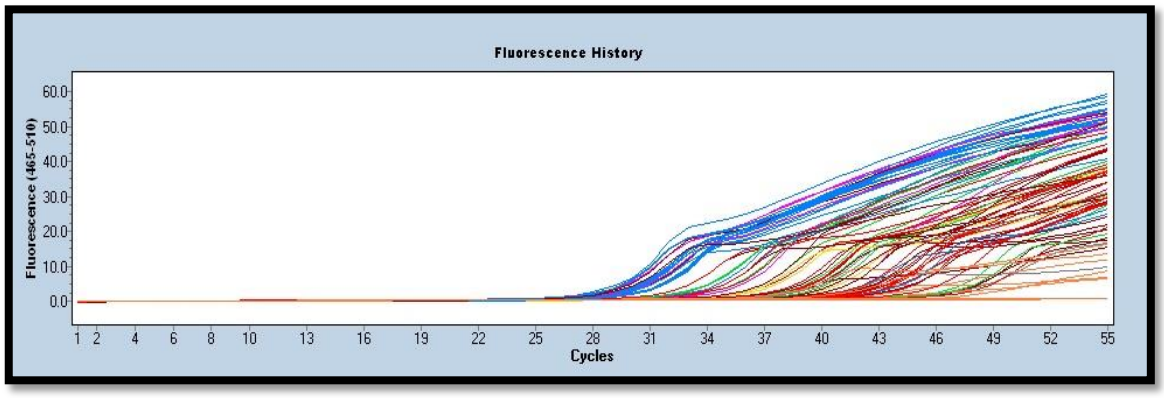

Figure E.10. Zooplankton amplification curve plot of 7. station obtained from Roche LightCycler 480 device

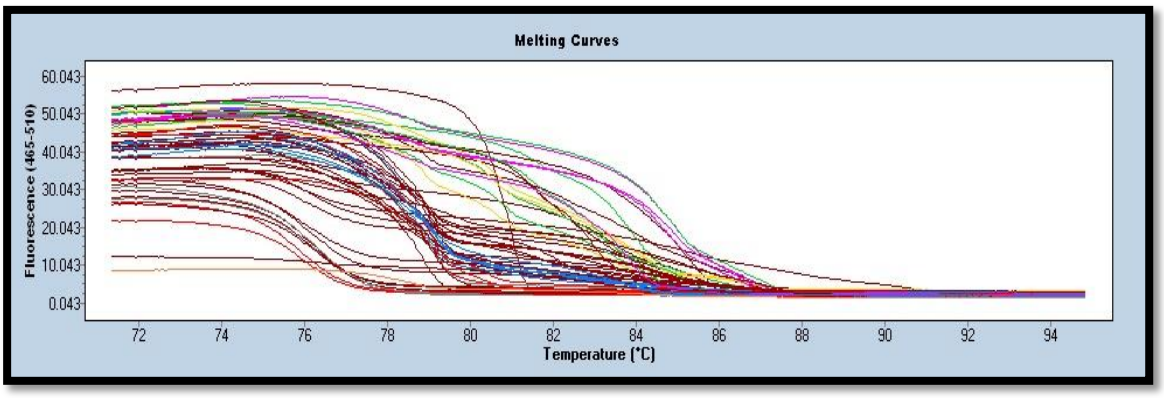

Figure E.11. Melting curve plot of fish species obtained from Roche LightCycler 480 device

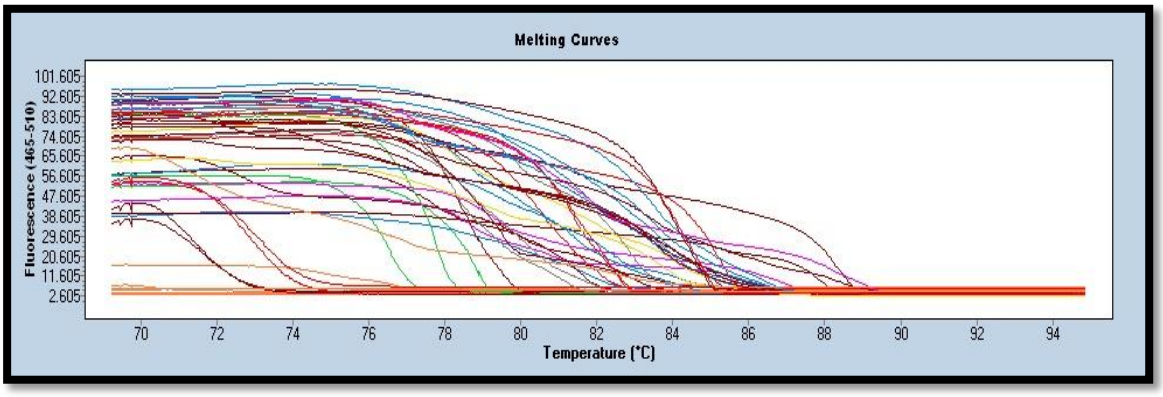

Figure E.12. Zooplankton melting curve plot of 1. station obtained from Roche LightCycler 480 device

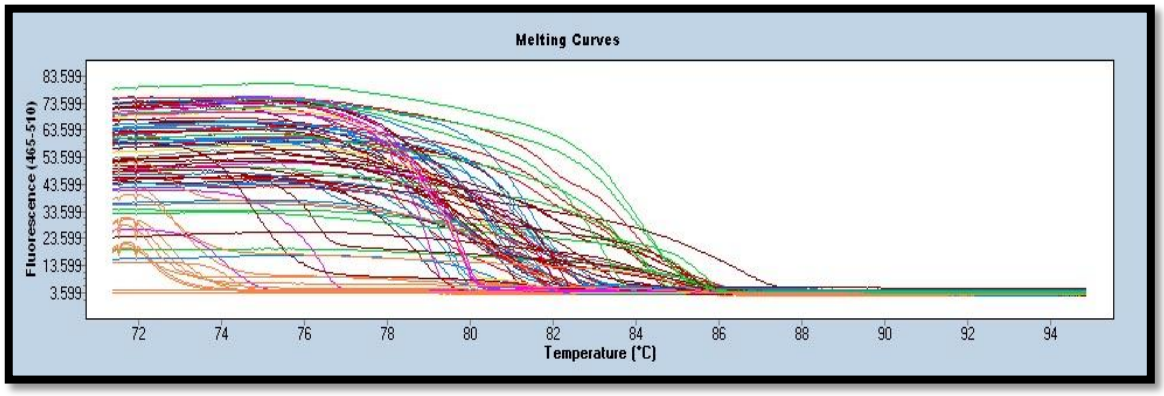

Figure E.13. Zooplankton melting curve plot of 2. station obtained from Roche LightCycler 480 device

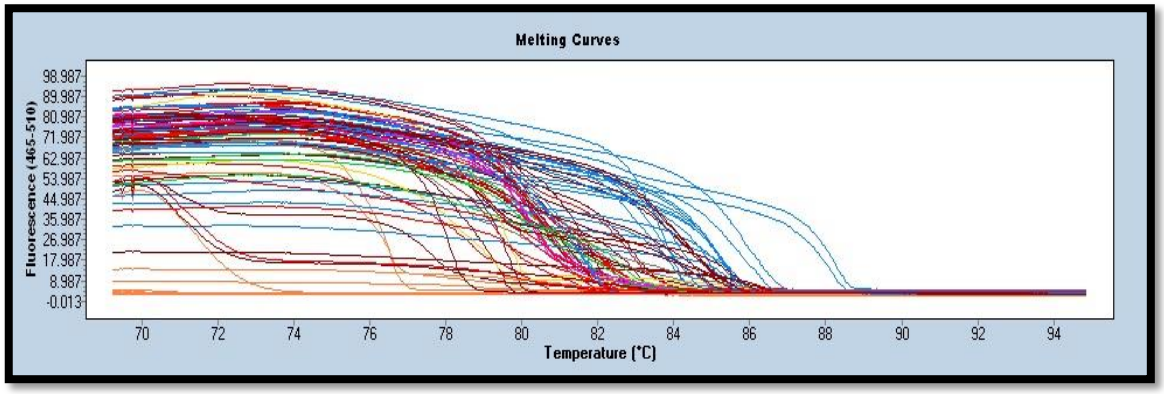

Figure E.14. Zooplankton melting curve plot of 3. station obtained from Roche LightCycler 480 device

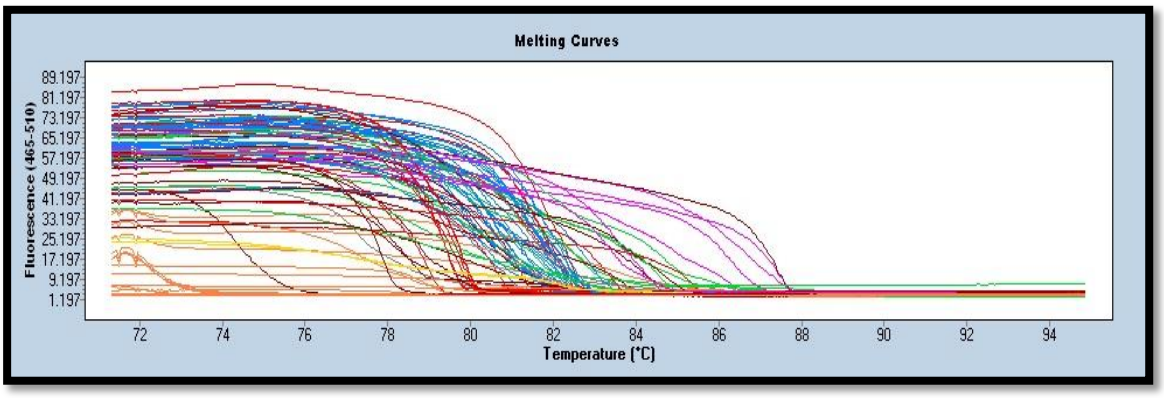

Figure E.15. Zooplankton melting curve plot of 4. station obtained from Roche LightCycler 480 device

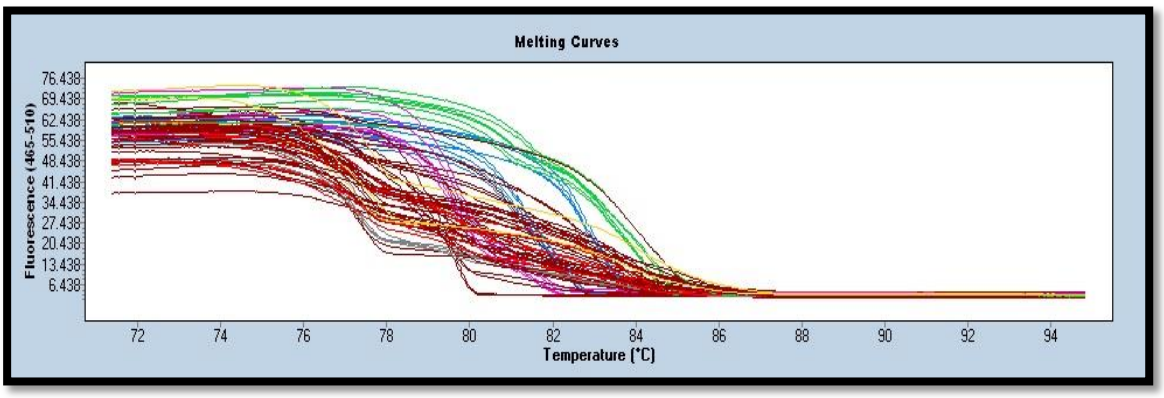

Figure E.16. Zooplankton melting curve plot of 5. station obtained from Roche LightCycler 480 device

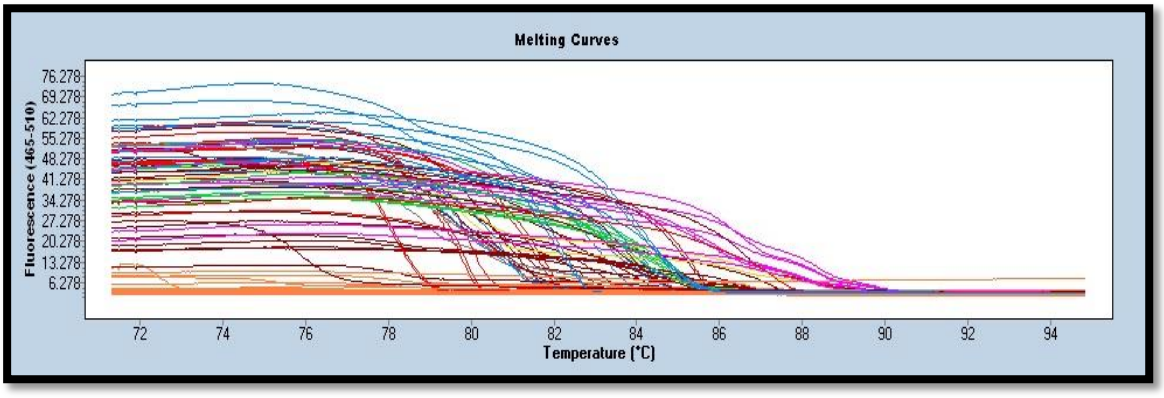

Figure E.17. Zooplankton melting curve plot of 5. station obtained from Roche LightCycler 480 device

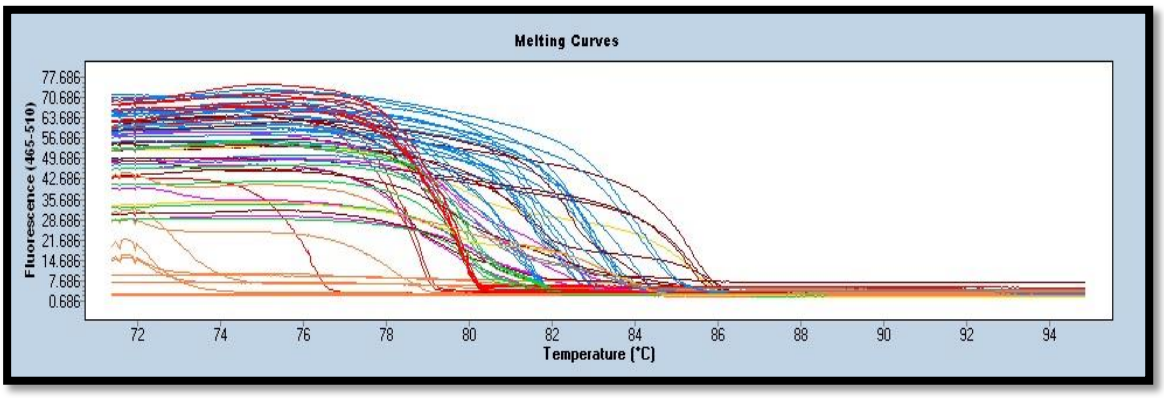

Figure E.18. Zooplankton melting curve plot of 6. station obtained from Roche LightCycler 480 device

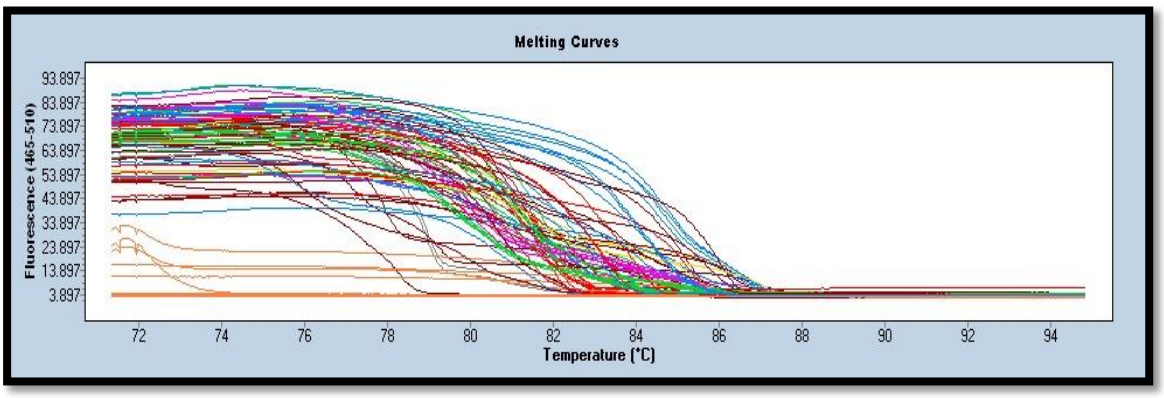

Figure E.19. Zooplankton melting curve plot of 6. station obtained from Roche LightCycler 480 device

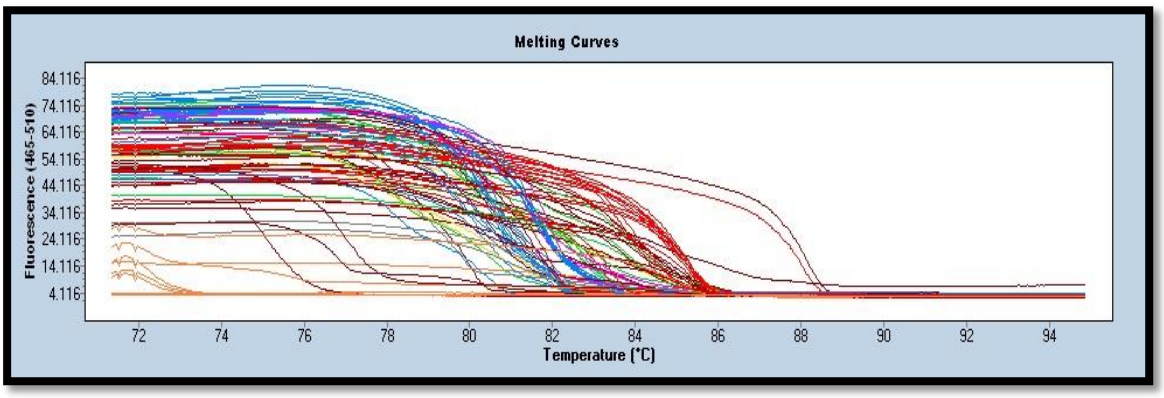

Figure E.20. Zooplankton melting curve plot of 7. station obtained from Roche LightCycler 480 device

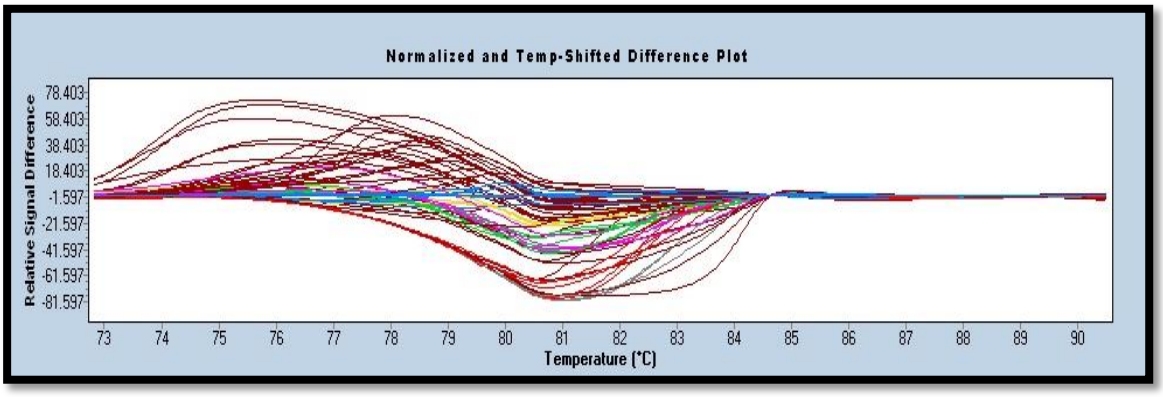

Figure E.21. Difference plot of fish species obtained from Roche LightCycler 480 device

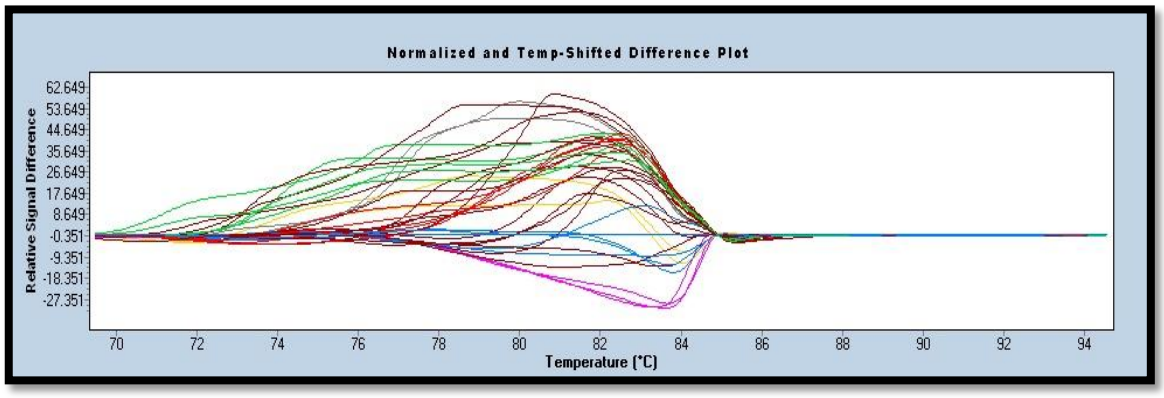

Figure E.22. Difference plot of 1. station obtained from Roche LightCycler 480 device

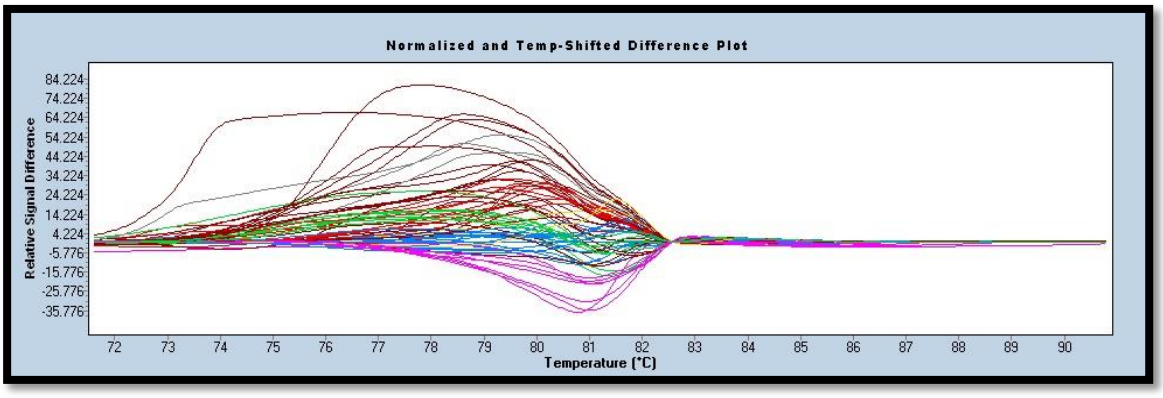

Figure E.23. Difference plot of 2. station obtained from Roche LightCycler 480 device

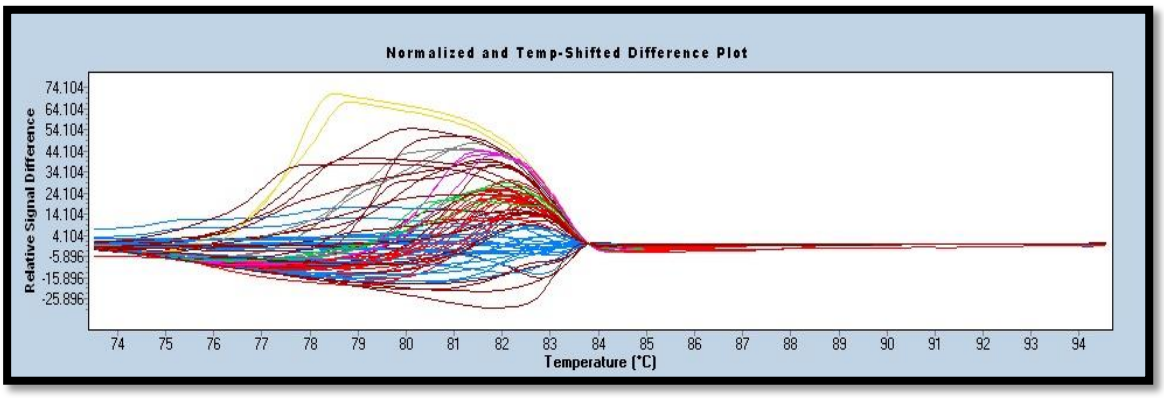

Figure E.24. Difference plot of 3. station obtained from Roche LightCycler 480 device

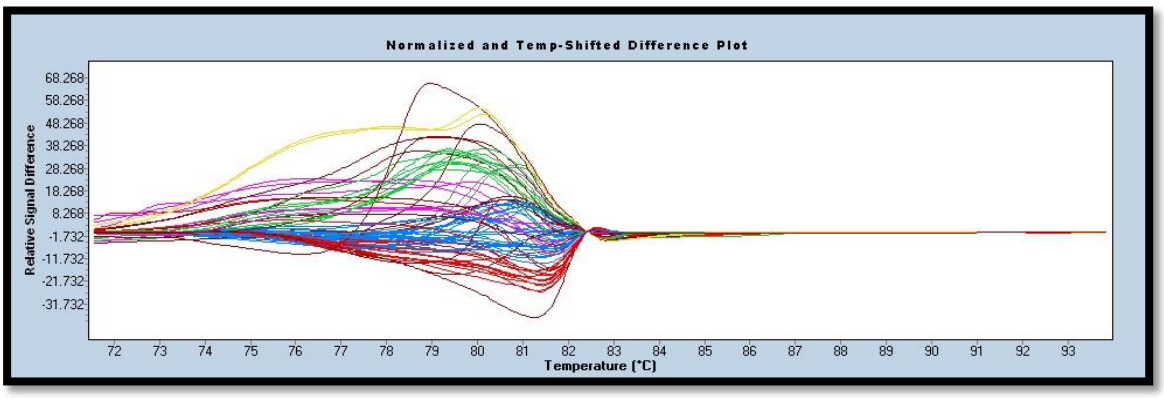

Figure E.25. Difference plot of 4. station obtained from Roche LightCycler 480 device

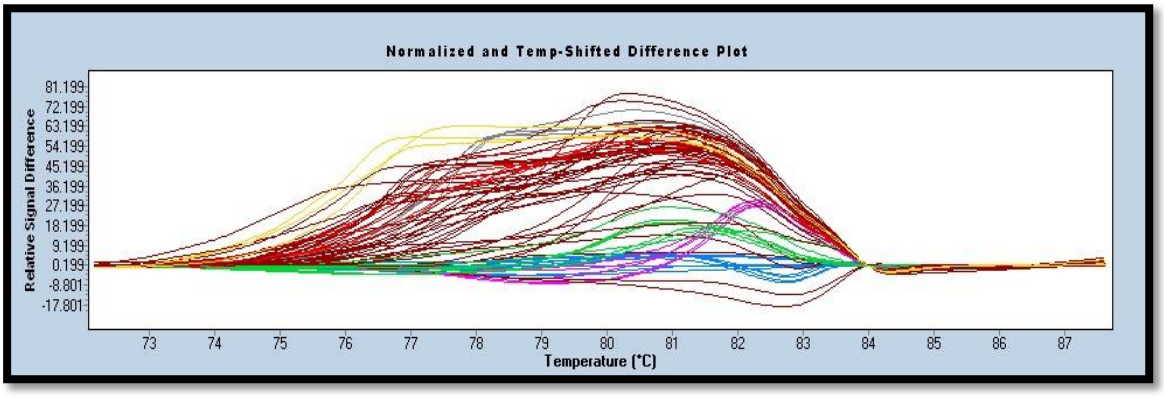

Figure E.26. Difference plot of 5. station obtained from Roche LightCycler 480 device

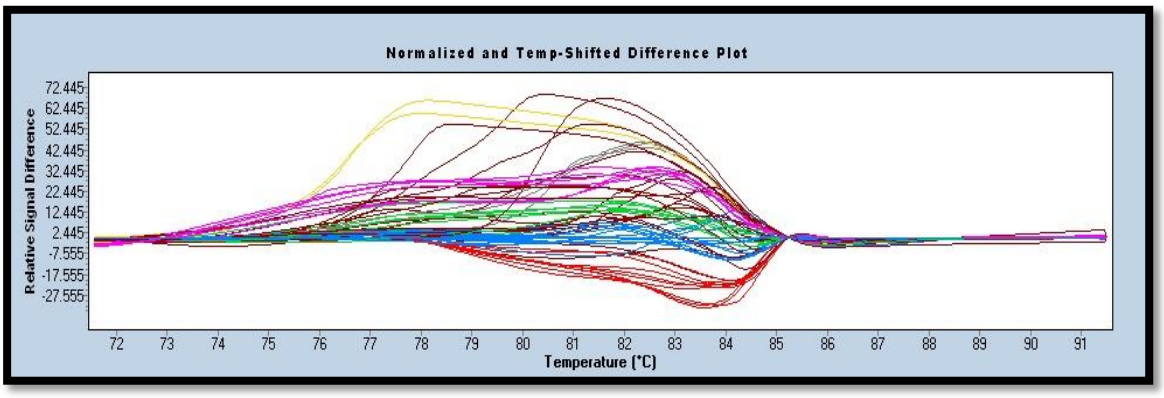

Figure E. 27. Difference plot of 5. station obtained from Roche LightCycler 480 device

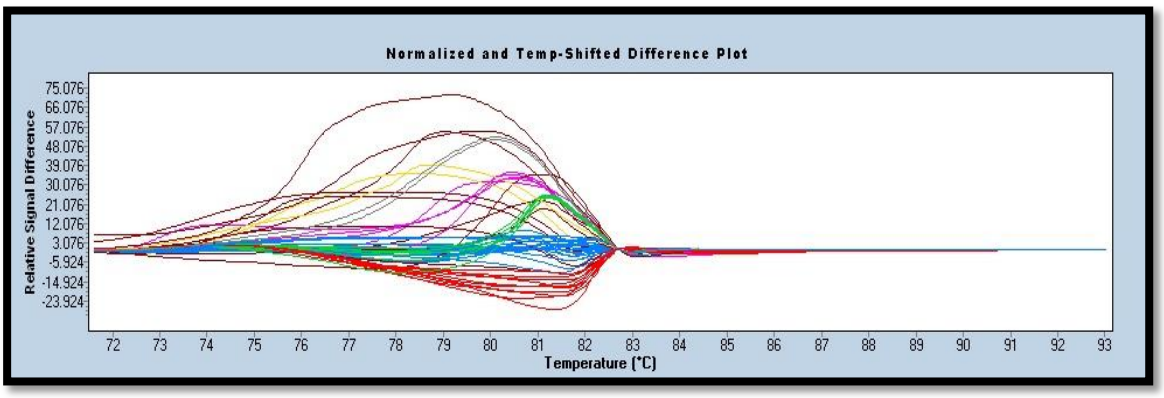

Figure E. 28. Difference plot of 6. station obtained from Roche LightCycler 480 device

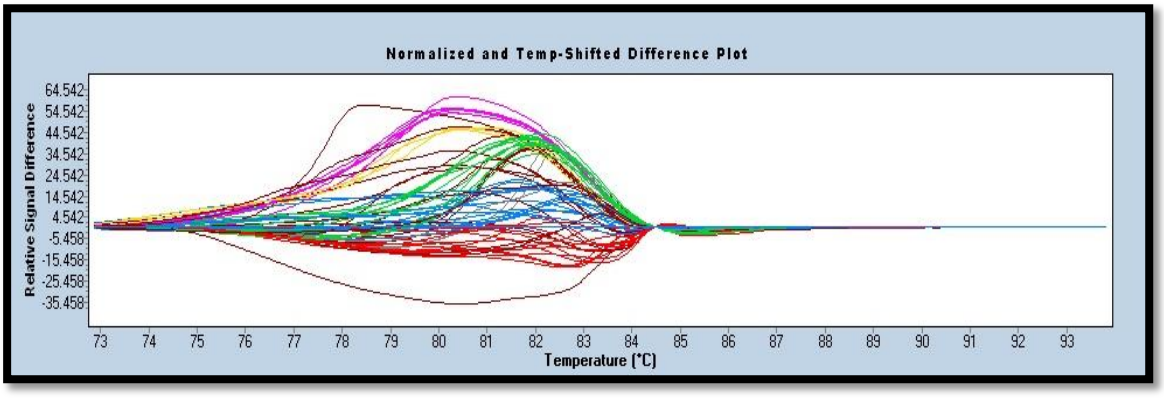

Figure E.29. Difference plot of 6. station obtained from Roche LightCycler 480 device

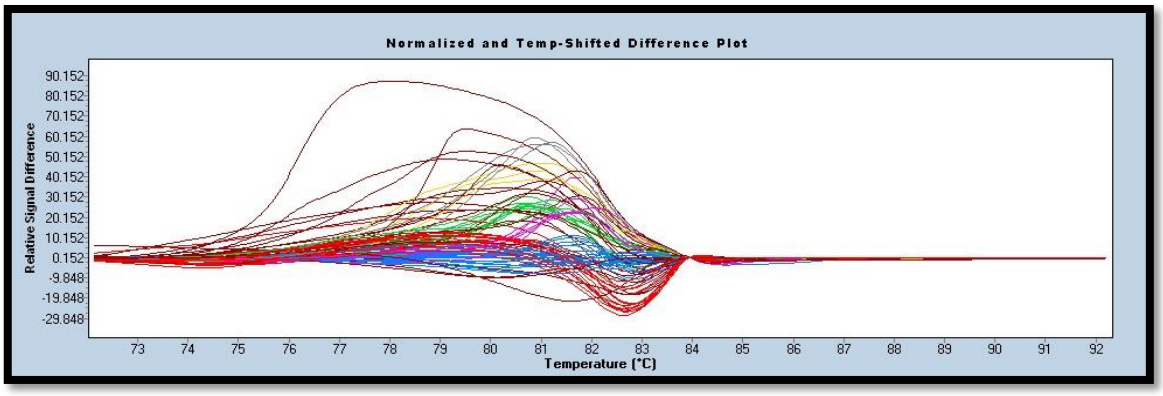

Figure E.30. Difference plot of 7. station obtained from Roche LightCycler 480 device

APPENDIX F: LIST OF IDENTIFIED ZOOPLANKTON

Table F.1. List of identified Zooplankton species

Code Name	Morhpholoical findings	Molecular findings	Accession
B3	Spicara flexuosa	Spicara maena	KP136713
bsex-071-app-1	Oikopleura dioica	unidentified Actinopteri	KP136548
bsex-071-app-3	Oikopleura dioica	unidentified Actinopteri	KP136549
bsex-071-CHS-3	Sagitta setosa	unidentifiedChaetognatha	KP136550
bsex-071-clapen-1	Penilia avirostris	Penilia avirostris	KP136551
bsex-071-clapen-2	Penilia avirostris	Penilia avirostris	KP136552
bsex-071-claspi-3	Evadne spinifera	unknown Crustacea	KP136553
bsex-071-cop-acl-1	Acartia sp.	unknown Crustacea	KP136554
bsex-071-copcale-1	Calanus euxinus	Calanuseuxinu	KP136555
bsex-071-cop-cenp-1	Centropages ponticus	unidentified Stomiiformes	KP136556
bsex-071-coppse-5	Pseudocalanus elongatus	Pseudocalanus elongatus	KP136558
bsex-071-cypd-1	Ctenophora (cydipid) sp1	unidentified Actinopteri	KP136559
bsex-071-ENGRA-EQ-3	Engraulis encrasicolus	Engraulis encrasicolus	KP136560
bsex-071-hydra-1	Hydromedusae	unidentified Eukaryota	KP136561
bsex-071-ost-1	Cirripedia	Amphibalanus improvisus	KP136562
bsex-071-ost-2	Cirripedia	Amphibalanus improvisus	KP136563
bsex-071-ost-3	Cirripedia	Amphibalanus improvisus	KP136564
C5	Scorpaena sp.	unidentified mullus sp.	KP136714
E1	Engraulis encrasicolus	Engraulis encrasicolus	KP136715
E2	Engraulis encrasicolus	unidentified Clupeiformes	KP136716
E3	Engraulis encrasicolus	Mullus barbatus	KP136717
E4	Engraulis encrasicolus	Engraulis encrasicolus	KP136718
E5	Engraulis encrasicolus	unidentified Clupeiformes	KP136719
F1	Merlangius merlangus euxinus	Merlangius merlangus	KP136720
F2	Merlangius merlangus	Merlangius merlangus	KP136721

	euxinus		
F3	Merlangius merlangus euxinu	Merlangius merlangus	KP136722
F4	Merlangius merlangus euxinus	Merlangius merlangus	KP136723
F5	Merlangius merlangus euxinus	Merlangius merlangus	KP136724
G1	Alosa pontica	Mesogobius batrachocephalus	KP136725
G2	Alosa pontica	Mesogobius batrachocephalus	KP136726
G3	Alosa pontica	Mesogobius batrachocephalus	KP136727
G4	Alosa pontica	Mesogobius batrachocephalus	KP136728
G5	Alosa pontica	Mesogobius batrachocephalus	KP136729
H1	Mullus barbatus	Mullus barbatus	KP136730
H2	Mullus barbatus	Mullus barbatus	KP136731
H4	Mullus barbatus	Mullus barbatus	KP136732
H5	Mullus barbatus	Mullus barbatus	KP136733
I1	Gobius niger	Mullus barbatus	KP136734
J1	Gaidropsarus mediterraneus	Gaidropsarus mediterraneus	KP136735
L1	Psetta maxima	Psetta maxima	KP136736
M1	Serranus hepatus	Serranus hepatus	KP136737
M2	Serranus hepatus	Serranus hepatus	KP136738
Q1	Neogobius melanostomus	Spicara maena	KP136739
Q3	Neogobius melanostomus	unidentified mesogobiussp.	KP136740
R1	Trachinus draco	Trachinus draco	KP136741
S1		Mesogobius batrachocephalus	KP136742

T1		Trionyx triunguis	KP136743
U1		Hymeniacidon sinapium	KP136744
X1	Gobius niger	Trachinus draco	KP136745
Y1		Trachinus draco	KP136746
2B-3	bentic species	Phyllophora sp.	KP136544
2B-5	bentic species	Cystoseira baccata	KP136545
2B-8	bentic species	unidentified Polychaeta	KP136546
2B-11	bentic species	unidentified Polychaeta	KP136539
2B-17	bentic species	unidentified Sabellida	KP136540
2B-20	bentic species	unidentified Polychaeta	KP136541
2B-22	bentic species	unidentified Sabellida	KP136542
2B-23	bentic species	unidentified Bangiophyceae	KP136543
2C-1	bentic species	unidentified Rhodophyta	KP136547
bsex-071-cop-cenp-3	Centropages ponticus	unidentified Actinopteri	KP136557
bsex-071-s		unidentifiedChaetognatha	KP136565
bsex-072-BRAC-SP1-1	Brachyura sp1	Cyclopoida sp.	KP136566
bsex-072-BRAC-SP1-3	Brachyura sp1	unidentified Brachyura	KP136567
bsex-072-BRAC-SP2-1	Brachyura sp2	unidentified Brachyura	KP136568
bsex-072-BRAC-SP3-1	Brachyura sp3	unidentified Xanthidae	KP136569
bsex-072-CHS-1	Sagitta setosa	unidentified Chaetognatha	KP136570
bsex-072-CHS-2	Sagitta setosa	unidentified Chaetognatha	KP136571
bsex-072-CHS-3	Sagitta setosa	unidentified Sagittoidea	KP136572
bsex-072-CLA-PEN-1	Penilia avirostris	Penilia avirostris	KP136573
bsex-072-CLA-PEN-2	Penilia avirostris	Penilia avirostris	KP136574
bsex-072-CLA-PEN-3	Penilia avirostris	Cyclopoida sp.	KP136575
bsex-072-COP-ACL-1	Acartia sp.	Acartia clausii	KP136576
bsex-072-COP-ACL-2	Acartia sp.	Acartia clausii	KP136577
bsex-072-COP-ACL-3	Acartia sp.	Acartia clausii	KP136578
bsex-072-COP-CAL-1	Calanus euxinus	Calanus euxinus	KP136579
bsex-072-COP-CAL-3	Calanus euxinus	Calanus euxinus	KP136580

bsex-072-COP-CENP-1	Centropages ponticus	unidentified Maxillopoda	KP136581
bsex-072-COP-PAR-1	Paracalanus parvus	unknown Copepoda	KP136582
bsex-072-engra-EQ-1	Engraulis encrasicolus egg	Engraulis encrasicolus	KP136583
bsex-072-engra-L-2	Engraulis encrasicolus larvae	Engraulis encrasicolus	KP136584
bsex-072-OST-1	Cirripedia	Amphibalanus improvisus	KP136585
bsex-072-OST-2	Cirripedia	Amphibalanus improvisus	KP136586
bsex-072-OST-3	Cirripedia	Amphibalanus improvisus	KP136587
bsex-117-chs-1	Sagitta setosa	unidentified Chaetognatha	KP136588
bsex-117-chs-2	Sagitta setosa	unidentified Chaetognatha	KP136589
bsex-117-chs-3	Sagitta setosa	unidentified Chaetognatha	KP136590
bsex-117-cla-ost-1	Cirripedia	Amphibalanus improvisus	KP136591
bsex-117-cla-ost-3	Cirripedia	Amphibalanus improvisus	KP136592
bsex-117-copaca-1	Acartia sp.	Acartia clausii	KP136593
bsex-117-copaca-2	Acartia sp.	Acartia clausii	KP136594
bsex-117-copaca-4	Acartia sp.	Acartia clausii	KP136595
bsex-117-copcal-2	Calanus euxinus	Calanus euxinus	KP136596
bsex-117-copcal-3	Calanus euxinus	Calanus euxinus	KP136597
bsex-117-cop-cenp-1	Centropages ponticus	unidentified Maxillopoda	KP136598
bsex-117-cop-cenp-2	Centropages ponticus	unidentified Gastropoda	KP136599
bsex-117-cop-cenp-3	Centropages ponticus	unidentified solenocerasp.	KP136600
bsex-117-cop-pse-1	Pseudocalanus elongatus	Pseudocalanus elongatus	KP136601
bsex-117-cop-pse-3	Pseudocalanus elongatus	Pseudocalanus elongatus	KP136602
bsex-117-engra-L-1	Engraulis encrasicolus larvae	Solenocera crassicornis	KP136603
BSEX-128-BIV-3	Bivalvia	unknown Bivalvia	KP136604
bsex-128-chs-1	Sagitta setosa	unidentifiedChaetognatha	KP136605
bsex-128-chs-2	Sagitta setosa	unidentifiedChaetognatha	KP136606
bsex-128-chs-3	Sagitta setosa	unidentifiedChaetognatha	KP136607

BSEX-128-CIRPL-1	Cirripedia Larvae	Amphibalanus improvisus	KP136608
BSEX-128-CIRPL-2	Cirripedia Larvae	Amphibalanus improvisus	KP136609
BSEX-128-CIRPL-3	Cirripedia Larvae	Amphibalanus improvisus	KP136610
BSEX-128-CLAPEN-1	Penilia avirostris	Penilia avirostris	KP136611
BSEX-128-CLAPEN-2	Penilia avirostris	Penilia avirostris	KP136612
BSEX-128-CLAPEN-3	Penilia avirostris	Penilia avirostris	KP136613
BSEX-128-CLA-SPI-1	Evadne spinifera	Amphibalanus improvisus	KP136614
BSEX-128-CLA-SPI-3	Evadne spinifera	Cyclopoida sp.	KP136615
BSEX-128-COPACA-1	Acartia sp.	Acartia clausii	KP136616
BSEX-128-COPACA-2	Acartia sp.	Acartia clausii	KP136617
BSEX-128-COPACA-3	Acartia sp.	Acartia clausii	KP136618
BSEX-128-COPCAL-1	Calanus euxinus	Calanus euxinus	KP136619
BSEX-128-COPCENP-1	Centropages ponticus	unidentified Gastropoda	KP136620
BSEX-128-COPCENP-2	Centropages ponticus	unidentified Maxillopoda	KP136621
BSEX-128-COPCENP-3	Centropages ponticus	unidentified Maxillopoda	KP136622
BSEX-128-COPPSE-1	Pseudocalanus elongatus	Pseudocalanus elongatus	KP136623
BSEX-128-COPPSE-2	Pseudocalanus elongatus	Pseudocalanus elongatus	KP136624
bsex-128-engra-egg-1	Engraulis encrasicolus	Engraulis encrasicolus	KP136625
bsex-128-engra-egg-3	Engraulis encrasicolus	Sitobion avenae	KP136626
bsex-128-engra-L-3	Engraulis encrasicolus	unidentified Engraulidae	KP136627
bsex-128-isop-1	Isopoda sp1	unidentified Malacostraca	KP136629
bsex-128-isop-2	Isopoda sp1	unidentified Malacostraca	P136630
BSEX-128-ISOP-L-3	Isopoda	unidentified Arthropoda	KP136628
CTD-23K-APP-1	Oikopleura dioica	Mullus barbatus	KP136631
CTD-23K-APP-3	Oikopleura dioica	unidentified Blenniidae	KP136632
CTD-23K-BIV-1	Bivalvia	Mesogobius batrachocephalus	KP136633
ctd-23k-brac-meg-L2-1	Brachyura Megalopa sp2	unidentified Brachyura	KP136634
ctd-23k-brac-meg-L2-2	Brachyura Megalopa sp2	unidentified Brachyura	KP136635
CTD-23K-BRAC-SP4-2	Brachyura sp4	unidentified Pilumnidae	KP136636
CTD-23K-BRAC-SP5-1	Brachyura sp5	Eriphia verrucosa	KP136637
ctd-23k-cepc-2	Cephalochordata	Branchiostoma lanceolatum	KP136638

ctd-23k-cepc-3	Cephalochordata	Branchiostoma lanceolatum	KP136639
CTD-23K-CIRPL-1	Cirripedia sp1	Amphibalanus improvisus	KP136640
CTD-23K-CIRPL-2	Cirripedia sp1	Amphibalanus improvisus	KP136641
CTD-23K-CIRPL-3	Cirripedia sp1	Amphibalanus improvisus	KP136642
CTD-23K-CIRPL-3-1	Cirripedia sp3	Euraphia depressa	KP136643
CTD-23K-CIRPL-3-2	Cirripedia sp3	unidentified euraphia sp.	KP136644
CTD-23K-CIRPL-L2-1	Cirripedia sp2	Amphibalanus improvisus	KP136645
CTD-23K-CLA-PEN-1	Penilia avirostris	Penilia avirostris	KP136646
CTD-23K-CLA-PEN-2	Penilia avirostris	Penilia avirostris	KP136647
CTD-23K-CLA-PEN-3	Penilia avirostris	Penilia avirostris	KP136648
CTD-23K-CLA-SPI-2	Evadne spinifera	Merlangius merlangus	KP136649
CTD-23K-CLA-SPI-3	Evadne spinifera	unidentified Gobiidae	KP136650
CTD-23K-COPACA-1	Acartia sp.	unidentified Calanoida	KP136651
CTD-23K-COPACA-2	Acartia sp.	Acartia clausii	KP136652
CTD-23K-COPACA-3	Acartia sp.	Acartia tonsa	KP136653
CTD-23K-COPCENP-3	Centropages ponticus	Mesogobius batrachocephalus	KP136654
CTD-23K-ENGRA-L-1	Engraulis encrasicolus	Upogebia pusilla	KP136655
CTD-23K-GAS-SP1-1	Gastropoda_sp1	unidentified Gastropoda	KP136656
CTD-23K-GAS-SP1-2	Gastropoda_s1	unidentified Gastropoda	KP136657
CTD-23K-GAS-SP1-3	Gastropoda_sp1	unidentified Gastropoda	KP136658
CTD-23K-GAS-SP2-1	Gastropoda_sp2	Rapana venosa	KP136659
CTD-23K-GAS-SP2-2	Gastropoda_sp2	Rapana venosa	KP136660
CTD-23K-GAS-SP2-3	Gastropoda_sp2	Rapana venosa	KP136661
ctd-23k-isop-L-1	Isopoda	unindetified Jakobida	KP136662
ctd-23k-poly-sp2-1	Polychaeta	unidentified Polychaeta	KP136663
ctd-23k-poly-sp2-3	Polychaeta	unidentified Polychaeta	KP136664
ctd-23k-poly-sp3-1	Polychaeta	unidentified Polychaeta	KP136665
ctd-23k-poly-sp3-2	Polychaeta	unidentified Polychaeta	KP136666
ctd-23k-poly-sp3-3	Polychaeta	unidentified Polychaeta	KP136667
ctd-23k-TL-Mb-1	Mullus barbatus	Mullus barbatus	KP136668
ctd-23k-TL-Mb-2	Mullus barbatus	Mullus barbatus	KP136669

CTD-23K-TL-SP1-1	Teleostei sp1	unidentified Blenniidae	KP136670
CTD-23K-TL-SP1-2	Teleostei sp1	unidentified Blenniidae	KP136671
CTD-23K-TL-SP1-3	Teleostei sp1	unidentified Blenniidae	KP136672
MAREX-COPCAL-2	Calanus euxinus	Calanus euxinus	KP136673
MAREX-ENGRA-EGG-3	Engraulis encrasicolus	Engraulis encrasicolus	KP136675
MAREX-ENGRA-L-1	Engraulis encrasicolus	Engraulis encrasicolus	KP136676
MAREX-ENGRA-L-2	Engraulis encrasicolus	Engraulis encrasicolus	KP136677
MAREX-ENGRA-L-3	Engraulis encrasicolus	Engraulis encrasicolus	KP136678
sile_acik-brac-megL1-1	Brachyura Megalopa sp1	unidentified Brachyura	KP136679
sile_acik-brac-sp6-2	Brachyura sp6	Eriphia verrucosa	KP136680
sile_acik-brac-sp6-3	Brachyura sp6	Eriphia verrucosa	KP136681
sile_acik-brac-sp7-1	Brachyura sp7	unidentified Xanthidae	KP136682
sile_acik-brac-sp7-2	Brachyura sp7	unidentified Xanthidae	KP136683
sile_acik-brac-sp7-3	Brachyura sp7	unidentified Xanthidae	KP136684
sile_acik-bry-cyp-L-1	Ctenophora (cydipid) sp1	Membranipora tenuis	KP136685
sile_acik-bry-cyp-L-2	Ctenophora (cydipid) sp1	Membranipora tenuis	KP136686
sile_acik-bry-cyp-L-3	Ctenophora (cydipid) sp1	Membranipora tenuis	KP136687
sile_acik-chs-1	Sagitta setosa	unidentified Chaetognatha	KP136688
sile_acik-chs-3	Sagitta setosa	unidentified Chaetognatha	KP136689
sile_acik-cirpL-1	Cirripedia Larvae	Amphibalanus improvisus	KP136690
sile_acik-cirpL-2	Cirripedia Larvae	Amphibalanus improvisus	KP136691
sile_acik-cirp-L2-1	Cirripedia Larvae sp2	Amphibalanus improvisus	KP136692
sile_acik-cirp-L2-2	Cirripedia Larvae sp2	Amphibalanus improvisus	KP136693
sile_acik-cirp-L2-3	Cirripedia Larvae sp2	Amphibalanus improvisus	KP136694
sile_acik-cla-evate-3	Evadne tergestina	Penilia avirostris	KP136695
sile_acik-cla-pen-1	Penilia avirostris	Penilia avirostris	KP136696
sile_acik-cla-pen-2	Penilia avirostris	Penilia avirostris	KP136697
sile_acik-cla-pen-3	Penilia avirostris	Penilia avirostris	KP136698
sile_acik-copaca-2	Acartia sp.	Acartia clausii	KP136699
sile_acik-copcenp-1	Centropages ponticus	unidentified Gastropoda	KP136700
sile_acik-copcenp-2	Centropages ponticus	unidentified Maxillopoda	KP136701

sile_acik-copcenp-3	Centropages ponticus	unidentified Gastropoda	KP136702
sile_acik-engra-egg-1	Engraulis encrasicolus	Engraulis encrasicolus	KP136703
sile_acik-gas-sp1-1	Gastropoda_sp1	unidentified Gastropoda	KP136704
sile_acik-gas-sp1-2	Gastropoda_sp1	unidentified Gastropoda	KP136705
sile_acik-gas-sp1-3	Gastropoda_sp1	unidentified Gastropoda	KP136706
sile_acik-gas-sp2-1	Gastropoda_sp2	Rapana venosa	KP136707
sile_acik-gas-sp2-2	Gastropoda_sp2	Rapana venosa	KP136708
sile_acik-gas-sp2-3	Gastropoda_sp2	Rapana venosa	KP136709
sile_acik-gas-sp3-1	Gastropoda_sp3	unidentified Gastropoda	KP136710
MAREX-COPCAL-3	Calanus euxinus	Calanus euxinus	KP136674

APPENDIX G: VISUAL OUTPUTS OF HRM SOFTWARE

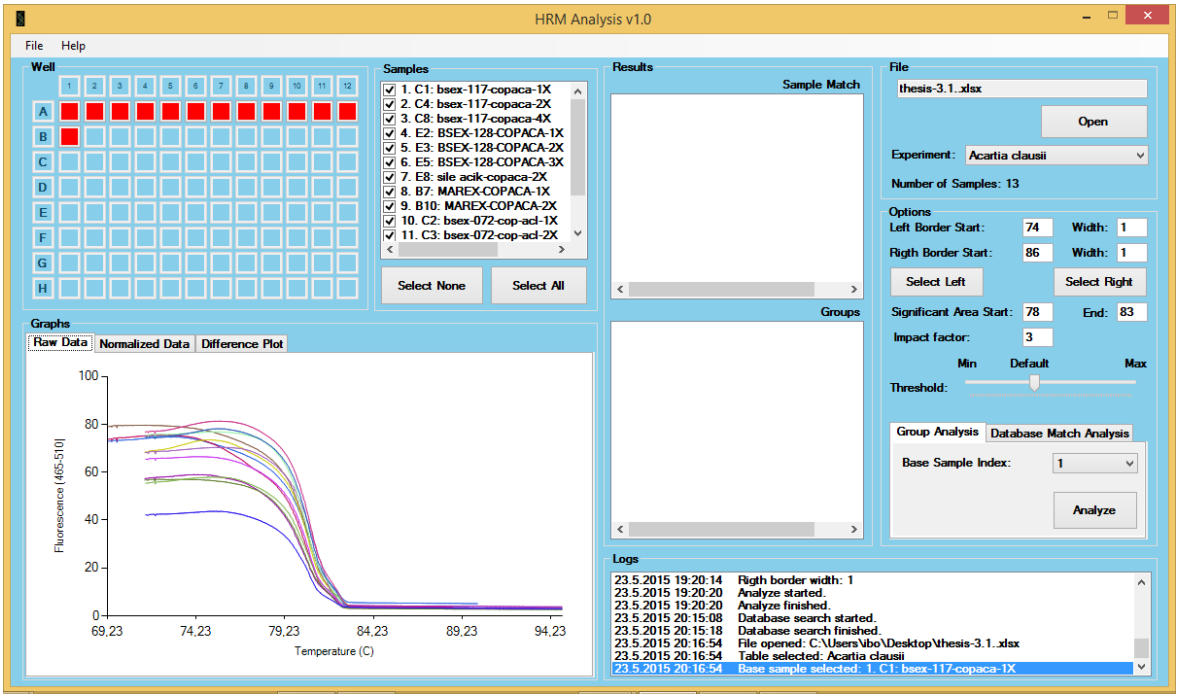

Figure G.1. Raw Melt curve data outputs of Acartia clausii organisms from HRM software

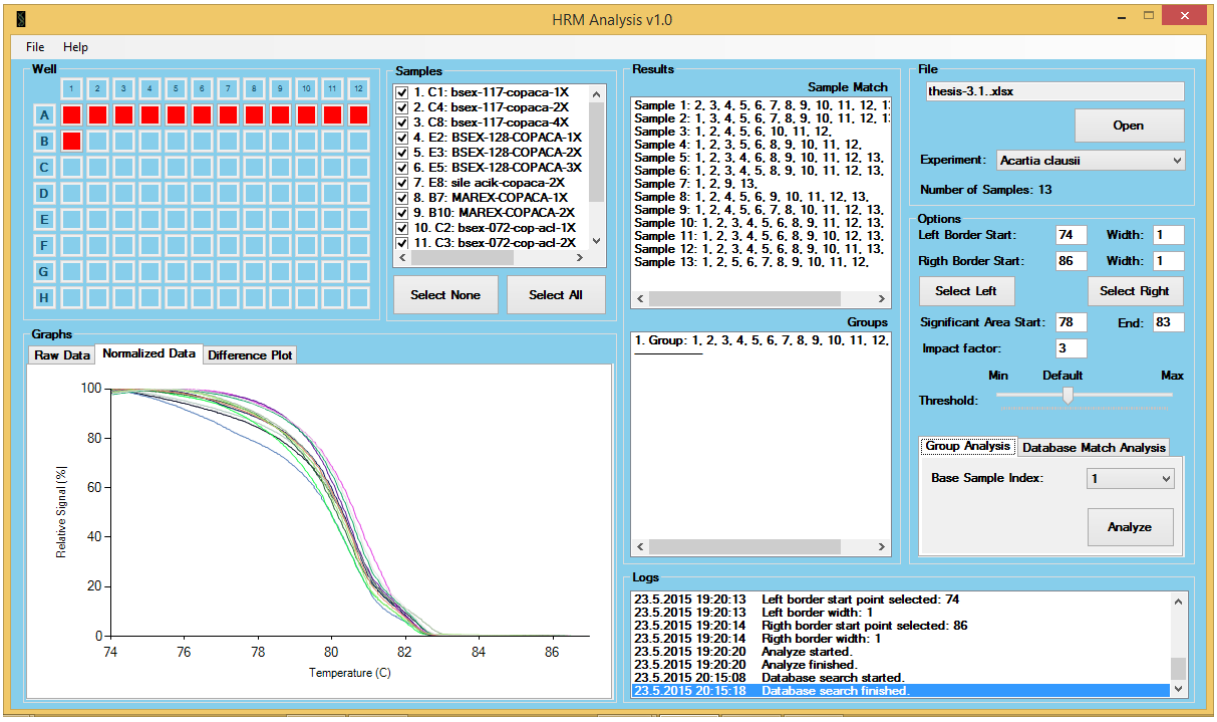

Figure G.2. Normalization and grouping outputs of Acartia clausii organisms from HRM software

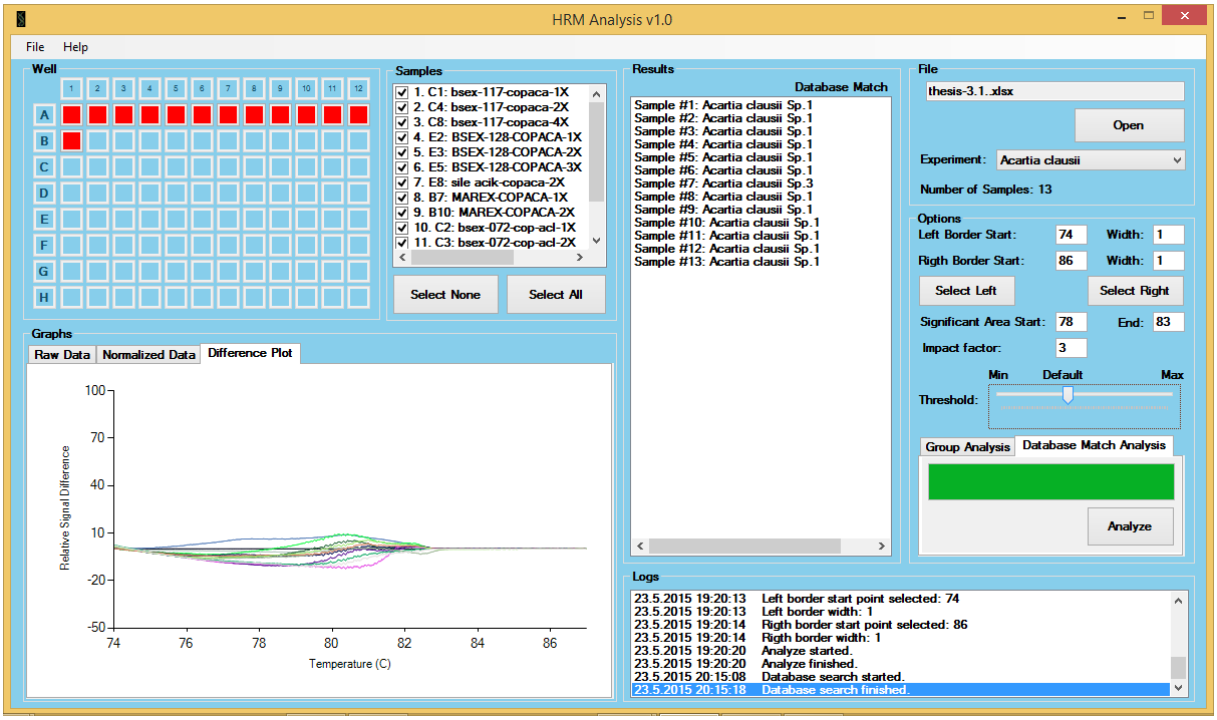

Figure G.3. Difference plot and database match analysis outputs of Acartia clausii organisms from HRM software

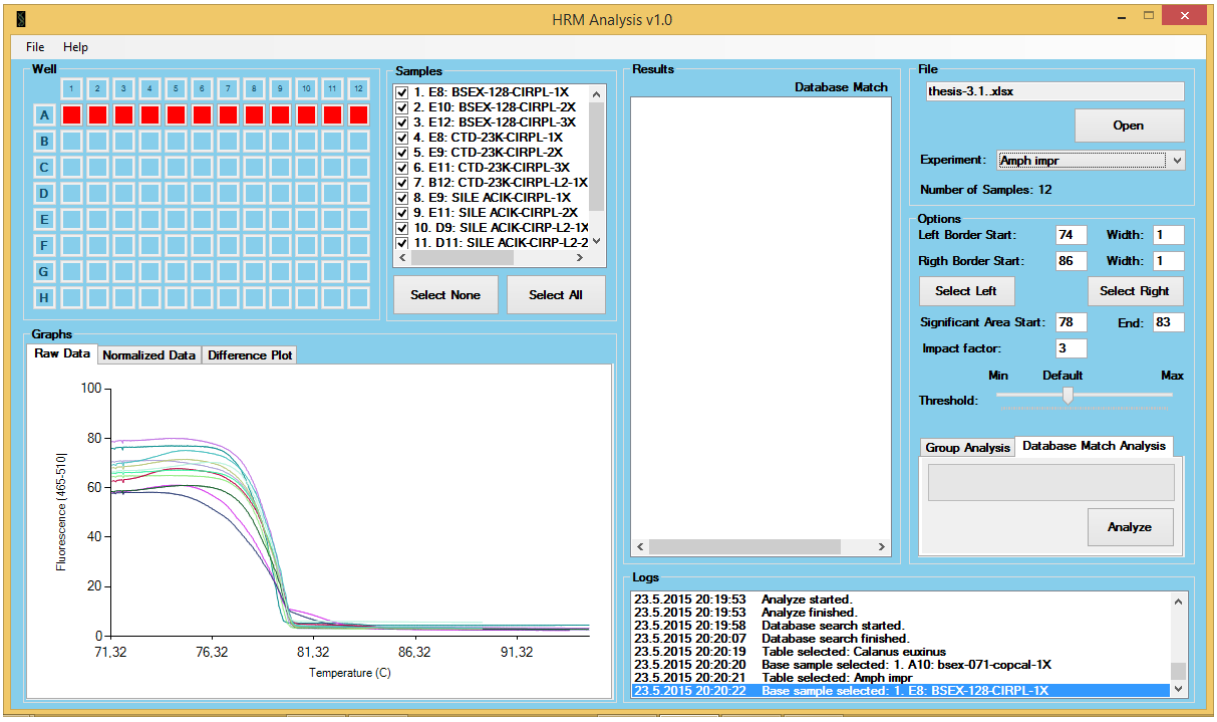

Figure G.4. Raw Melt curve data outputs of Amphibalanus improvises organisms from HRM software

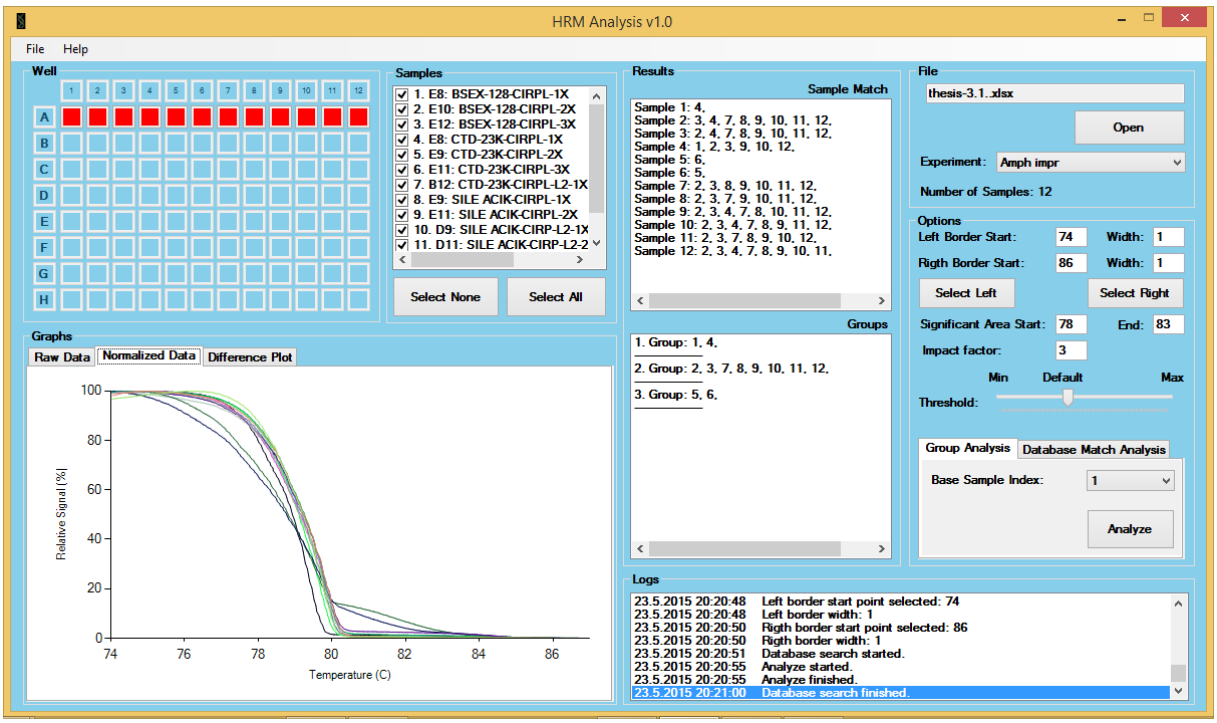

Figure G.5. Normalization and grouping outputs of Amphibalanus improvises organisms from HRM software

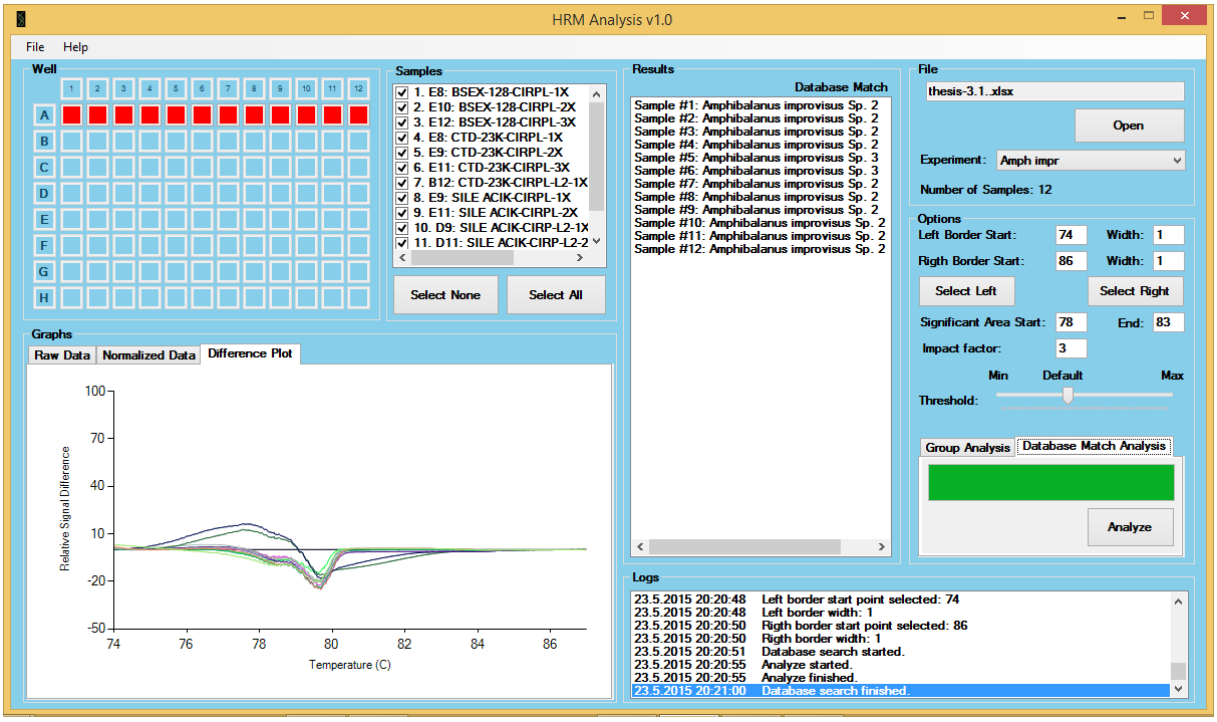

Figure G.6. Difference plot and database match analysis outputs of Amphibalanus improvises organisms from HRM software

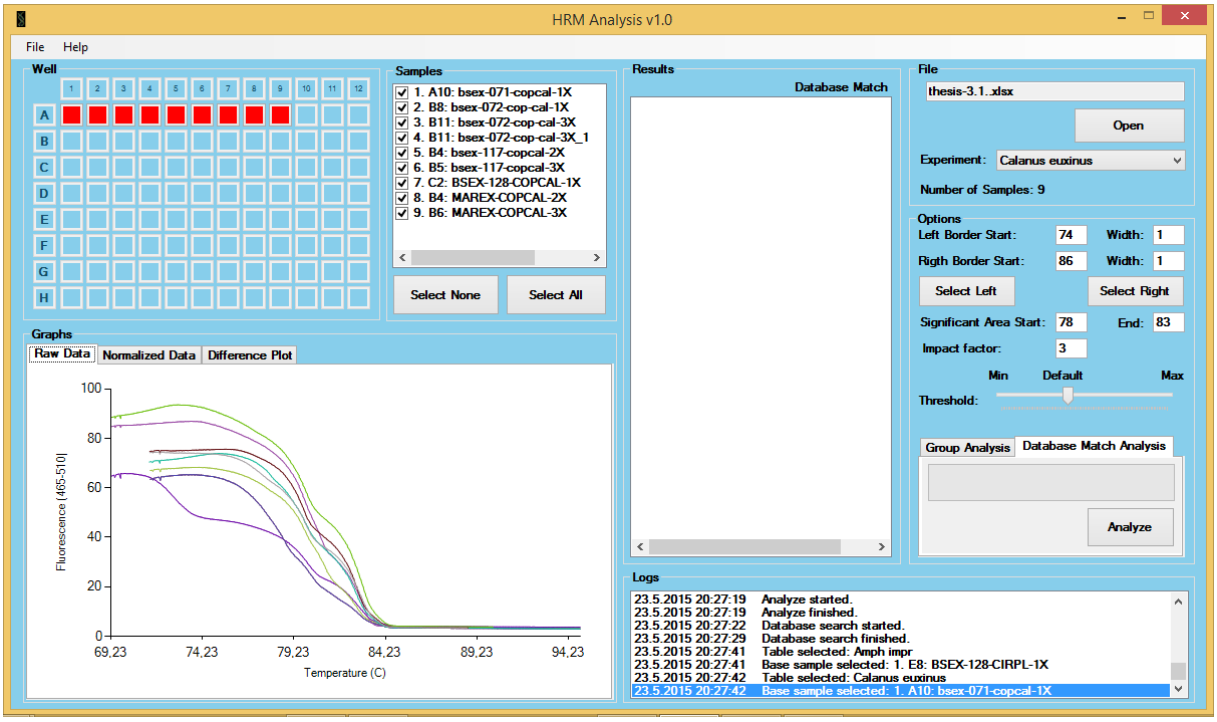

Figure G.7. Raw Melt curve data outputs of Calanus euxinus organisms from HRM software

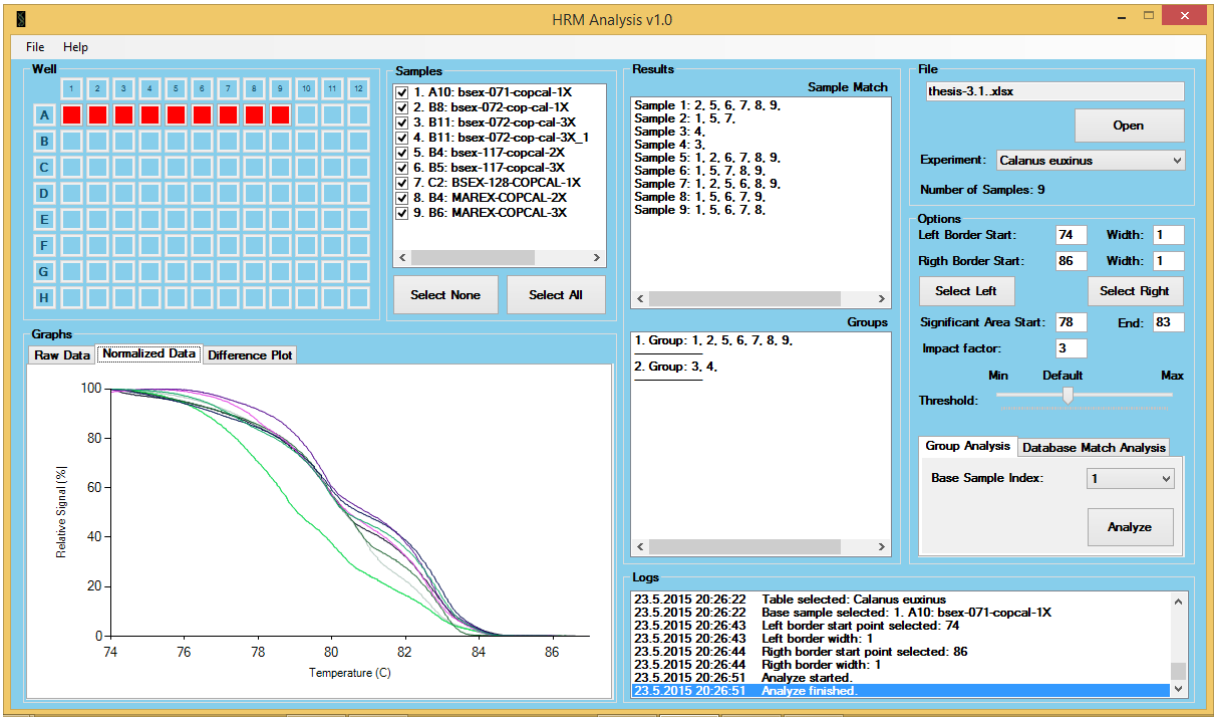

Figure G.8. Normalization and grouping outputs of Calanus euxinus organisms from HRM software

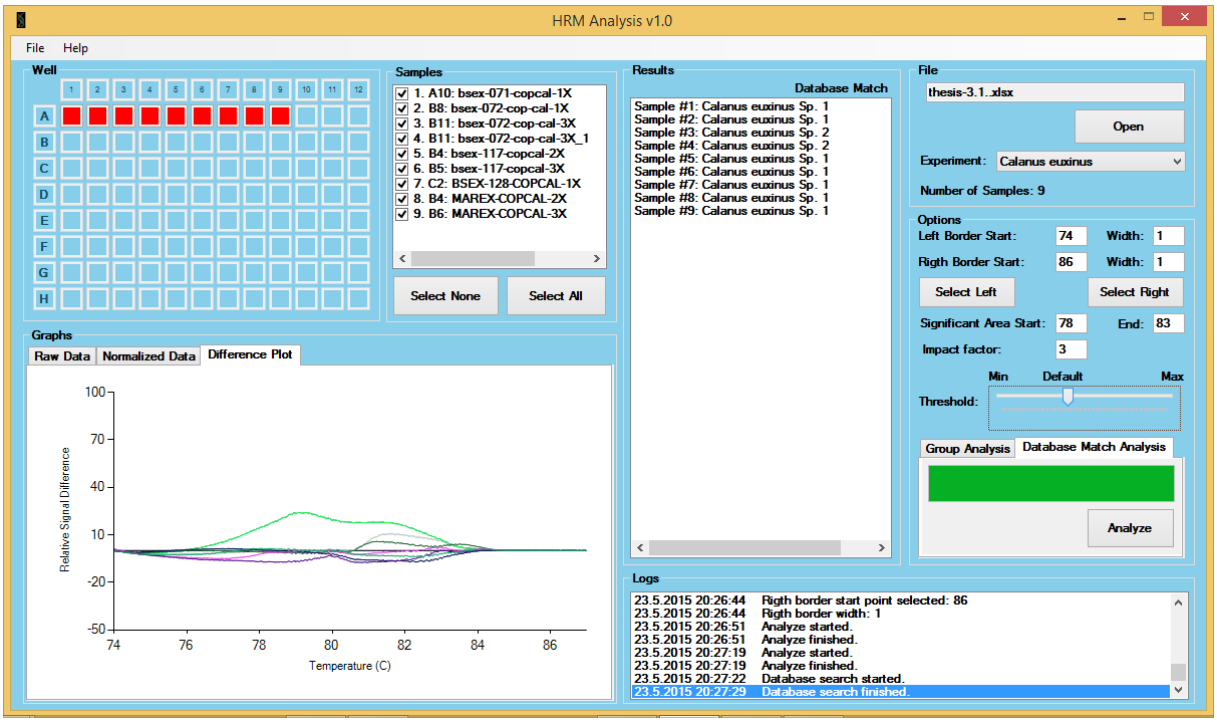

Figure D.9. Difference plot and database match analysis outputs of Calanus euxinus organisms from HRM software

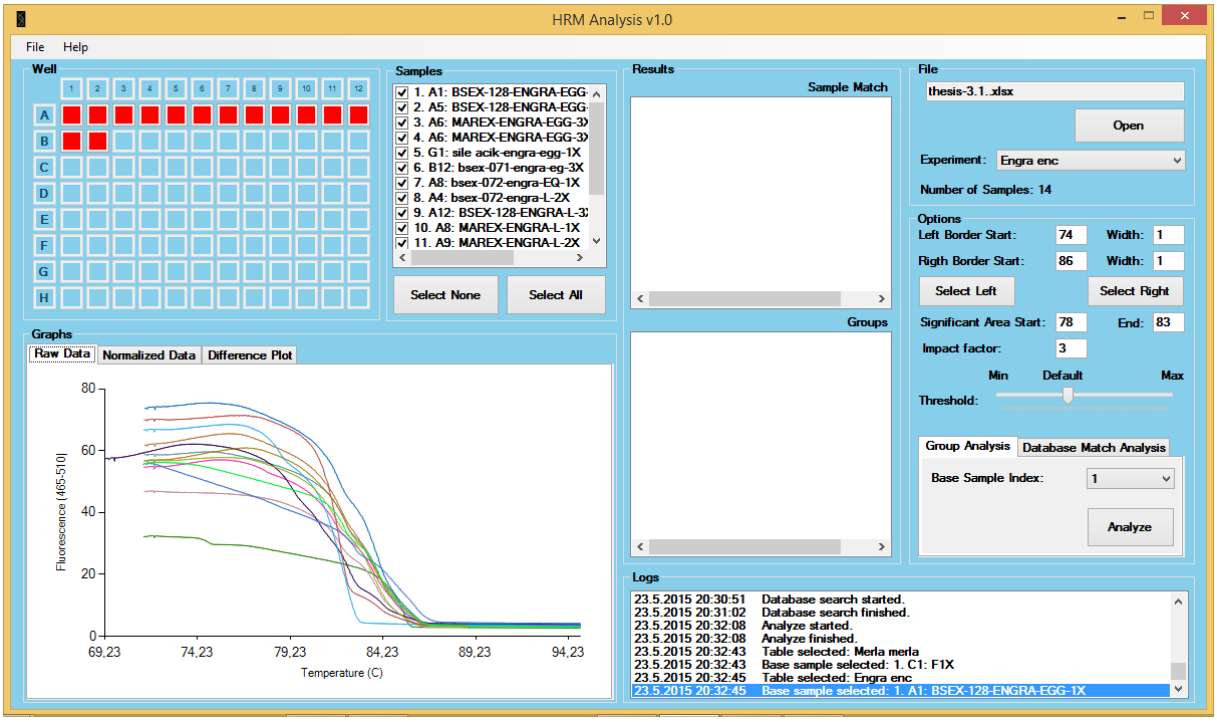

Figure G.10. Raw Melt curve data outputs of Engraulis encrasicolus organisms from HRM software

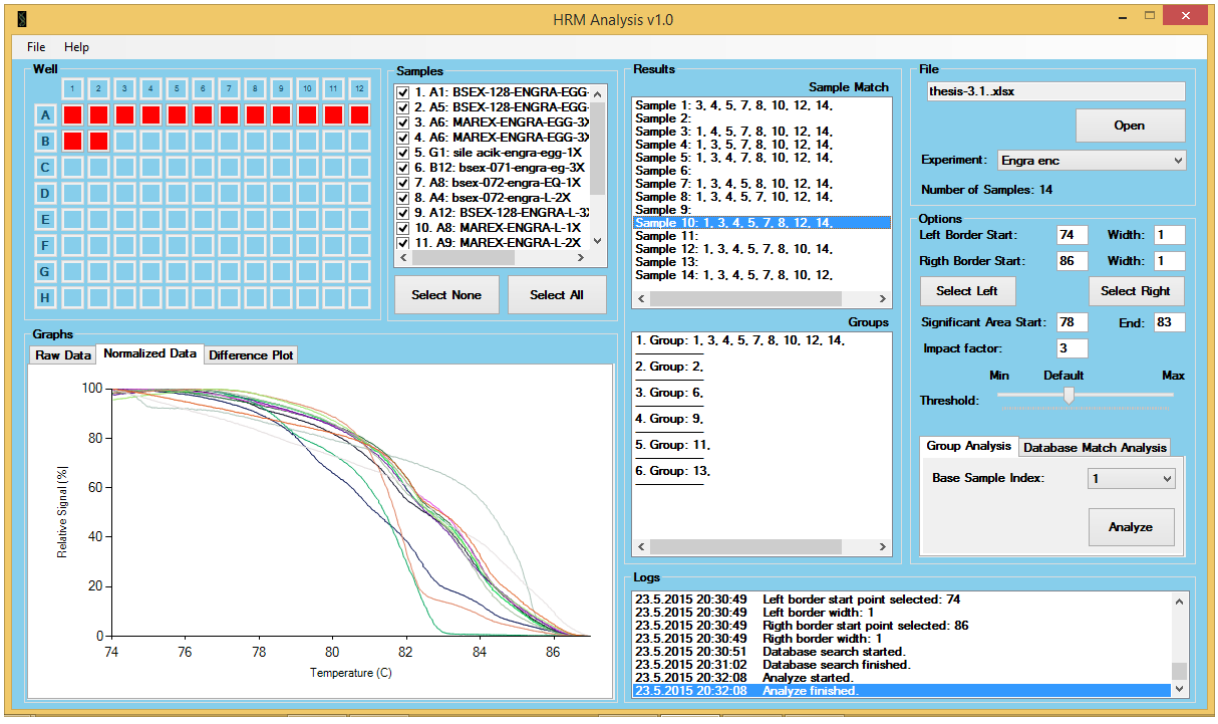

Figure G.11. Normalization and grouping outputs of Engraulis encrasicolus organisms from HRM software

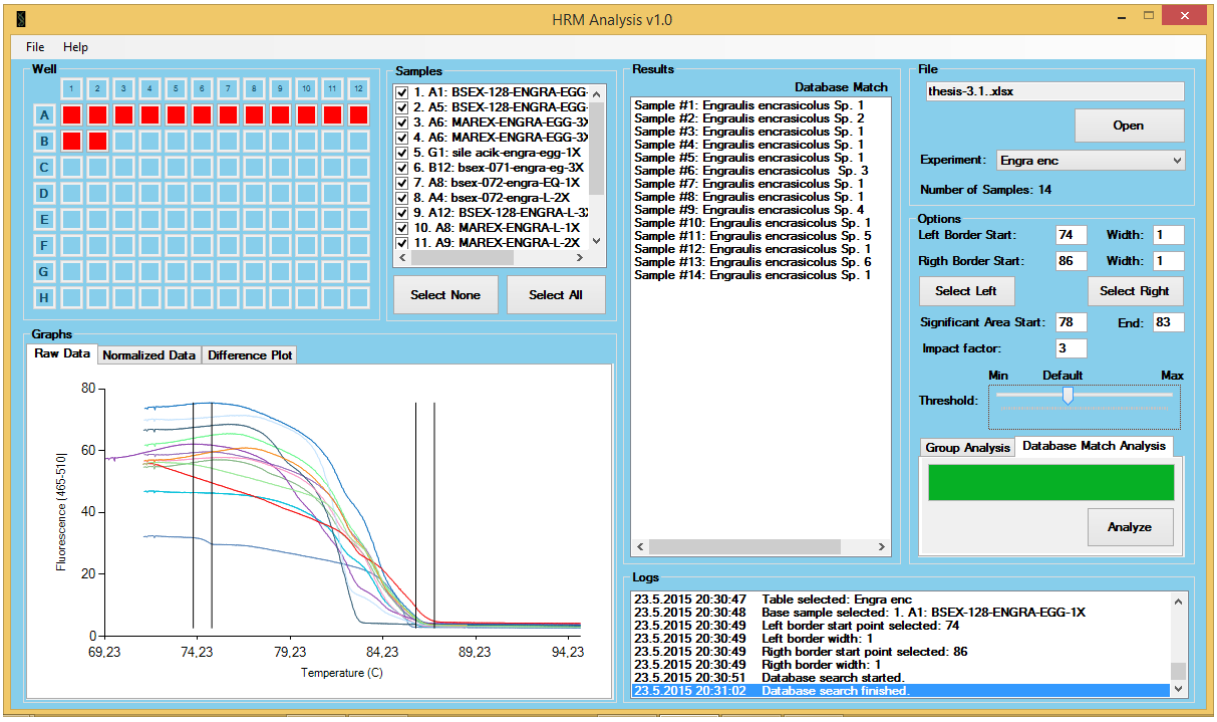

Figure G.12. Difference plot and database match analysis outputs of Engraulis encrasicolus organisms from HRM software

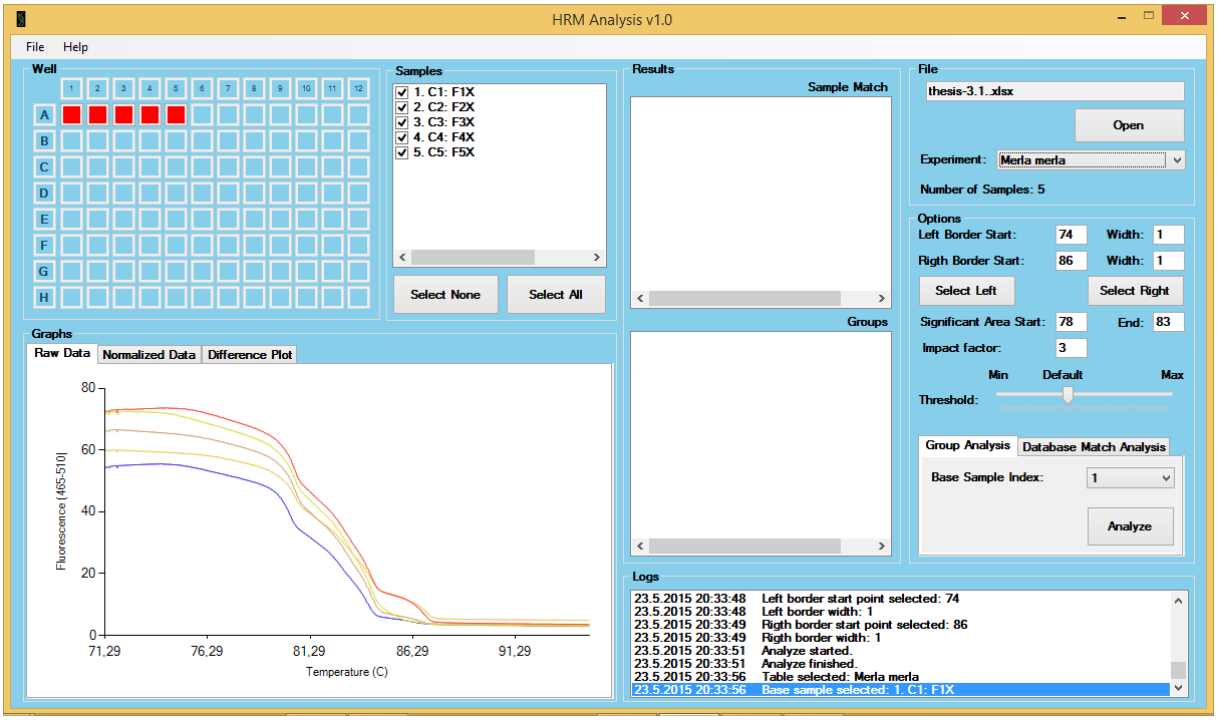

Figure G.13. Raw Melt curve data outputs of Merlangius merlangus organisms from HRM software

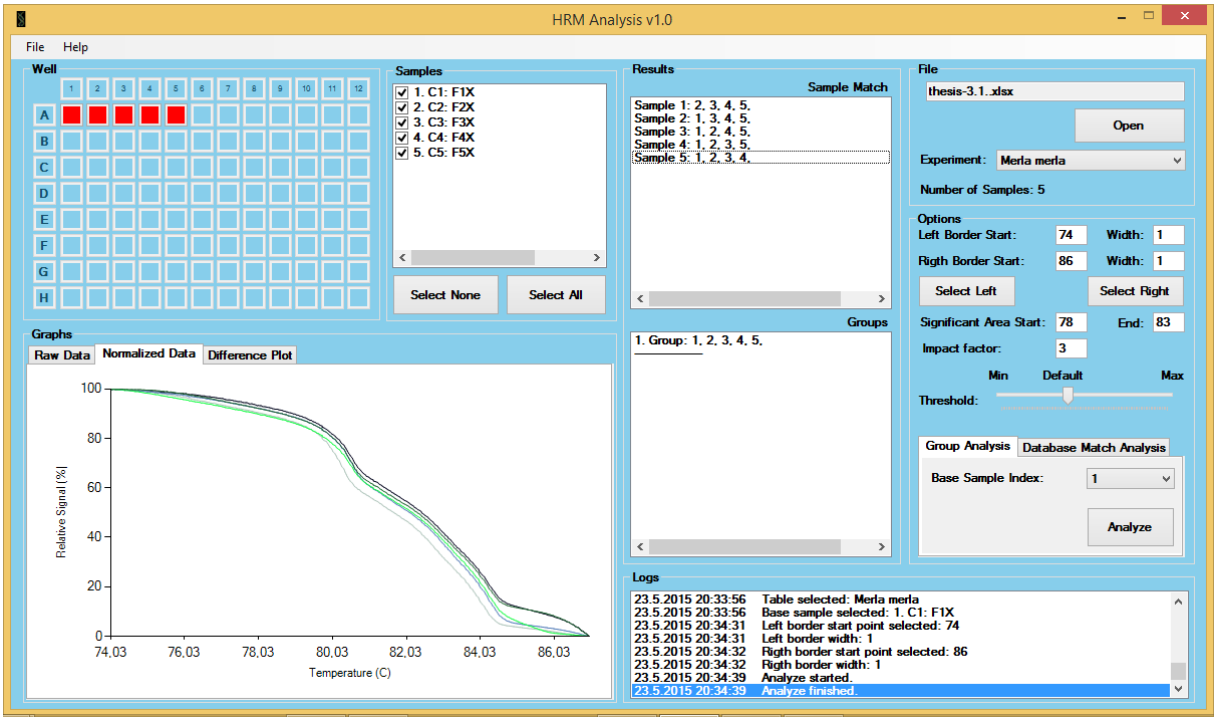

Figure G.14. Normalization and grouping outputs of Merlangius merlangus organisms from HRM software

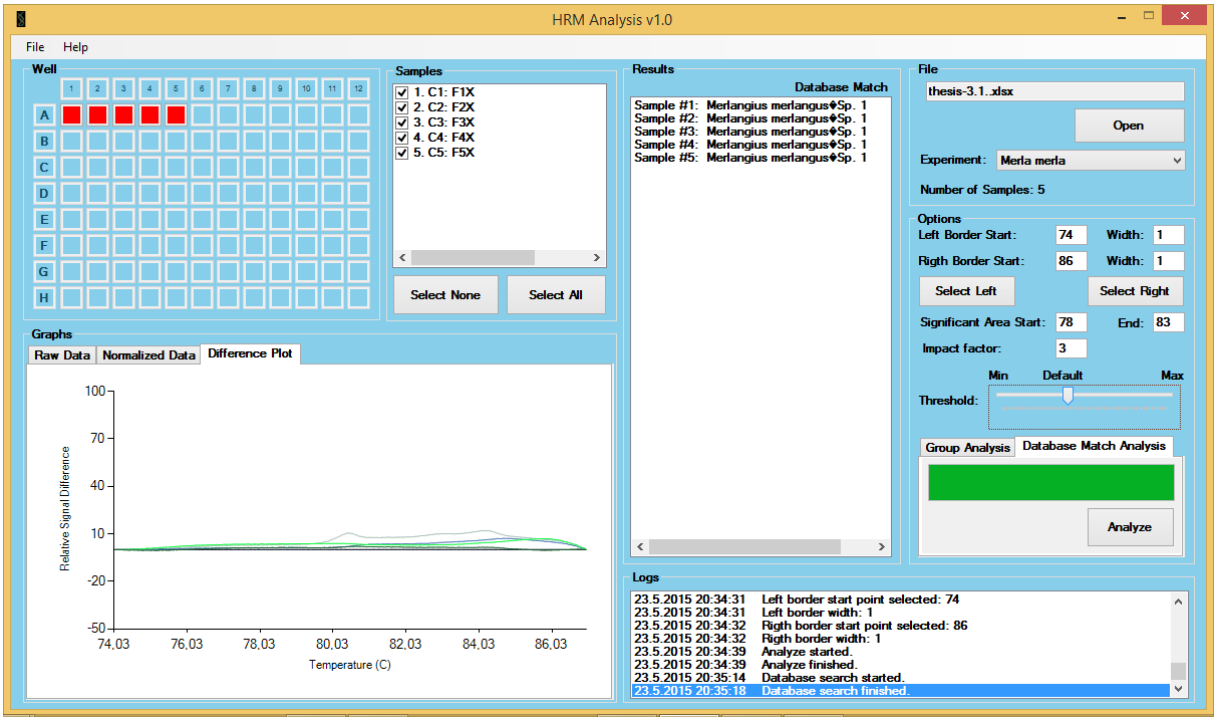

Figure G.15. Difference plot and database match analysis outputs of Merlangius merlangus organisms from HRM software

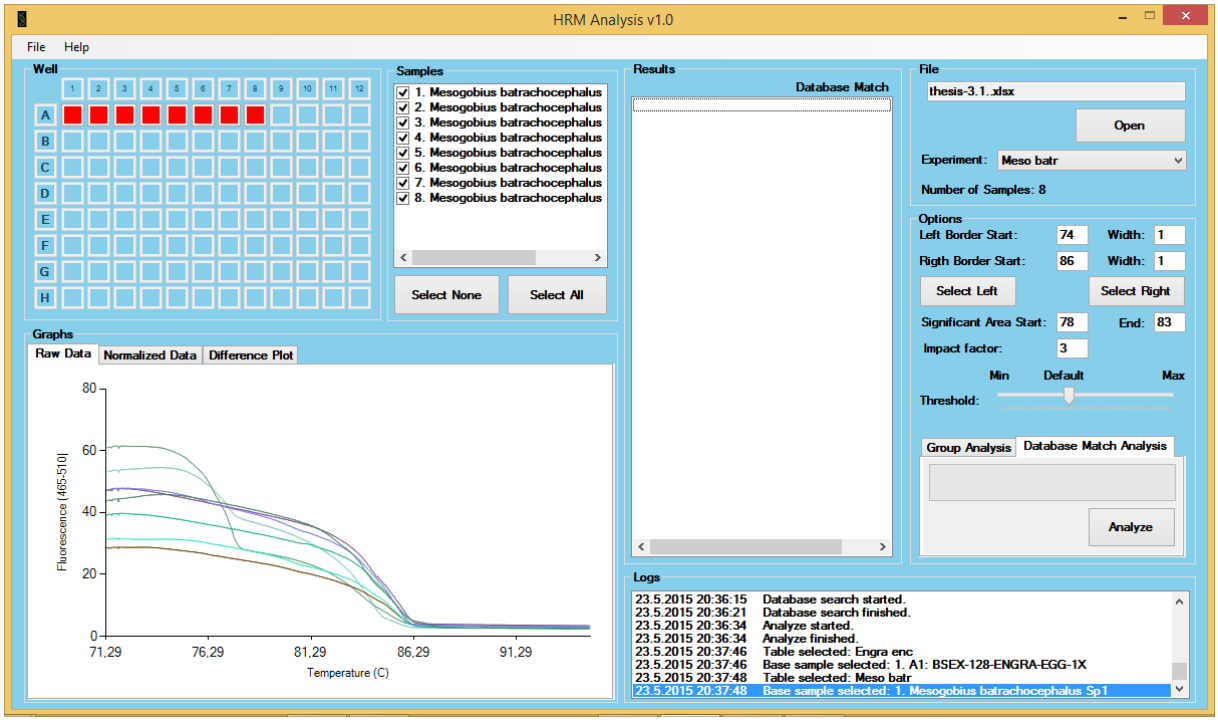

Figure G.16. Raw Melt curve data outputs of Mesogobius batrachocephalus organisms from HRM software

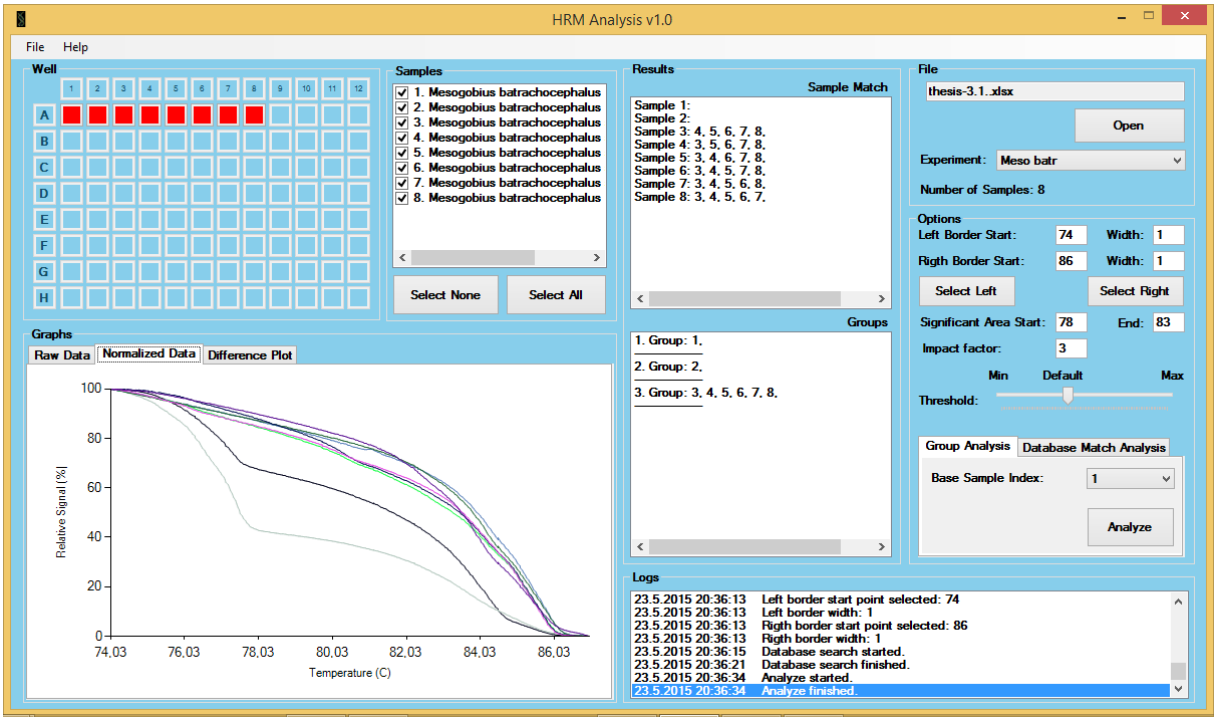

Figure G.17. Normalization and grouping outputs of Mesogobius batrachocephalus organisms from HRM software

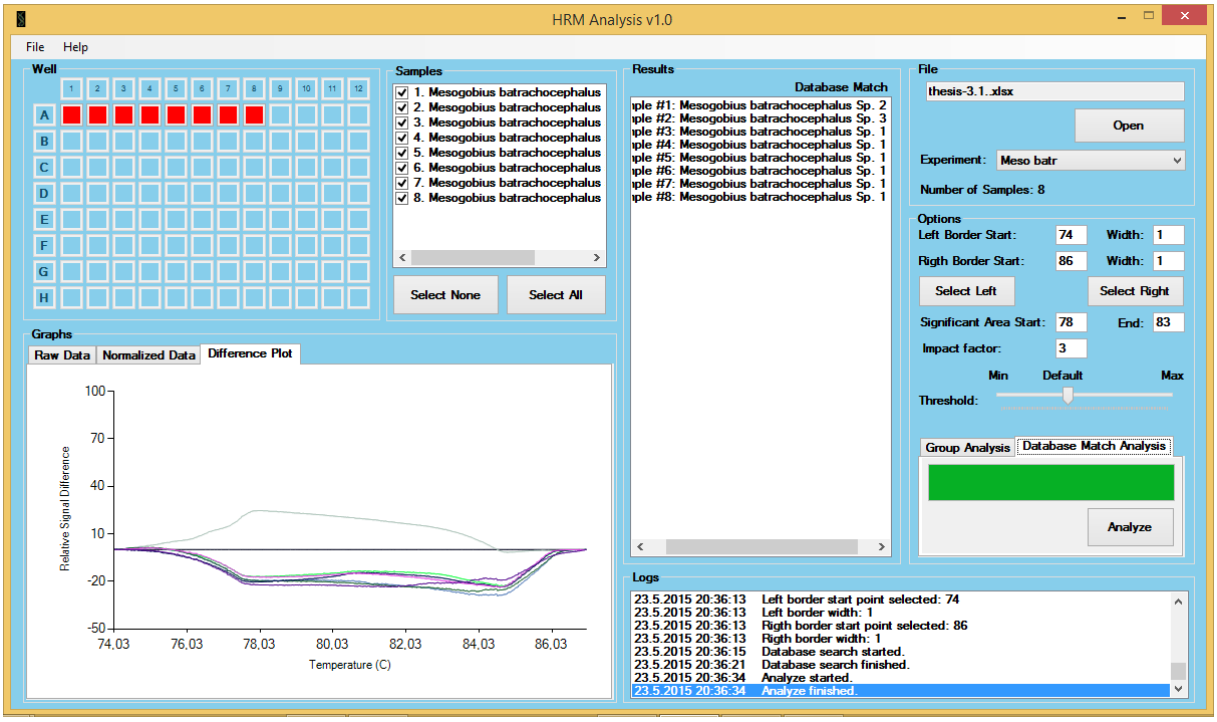

Figure G.18. Difference plot and database match analysis outputs of Mesogobius batrachocephalus organisms from HRM software

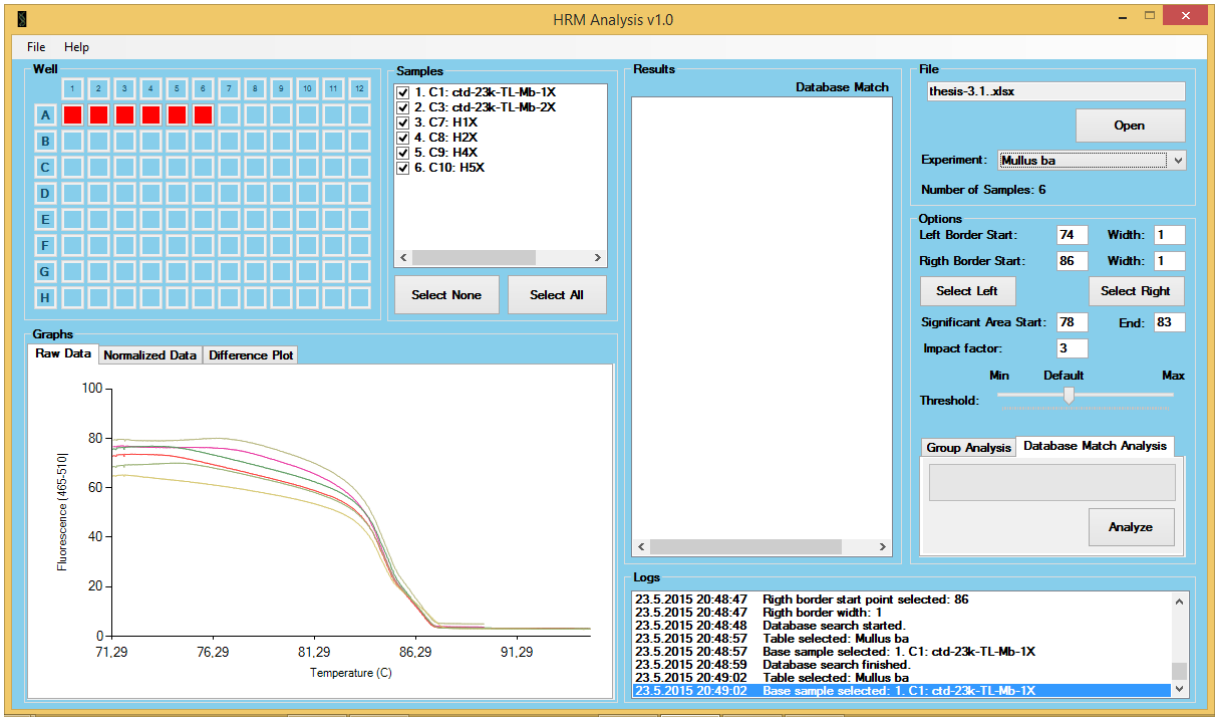

Figure G.19. Raw Melt curve data outputs of Mullus barbatus organisms from HRM software

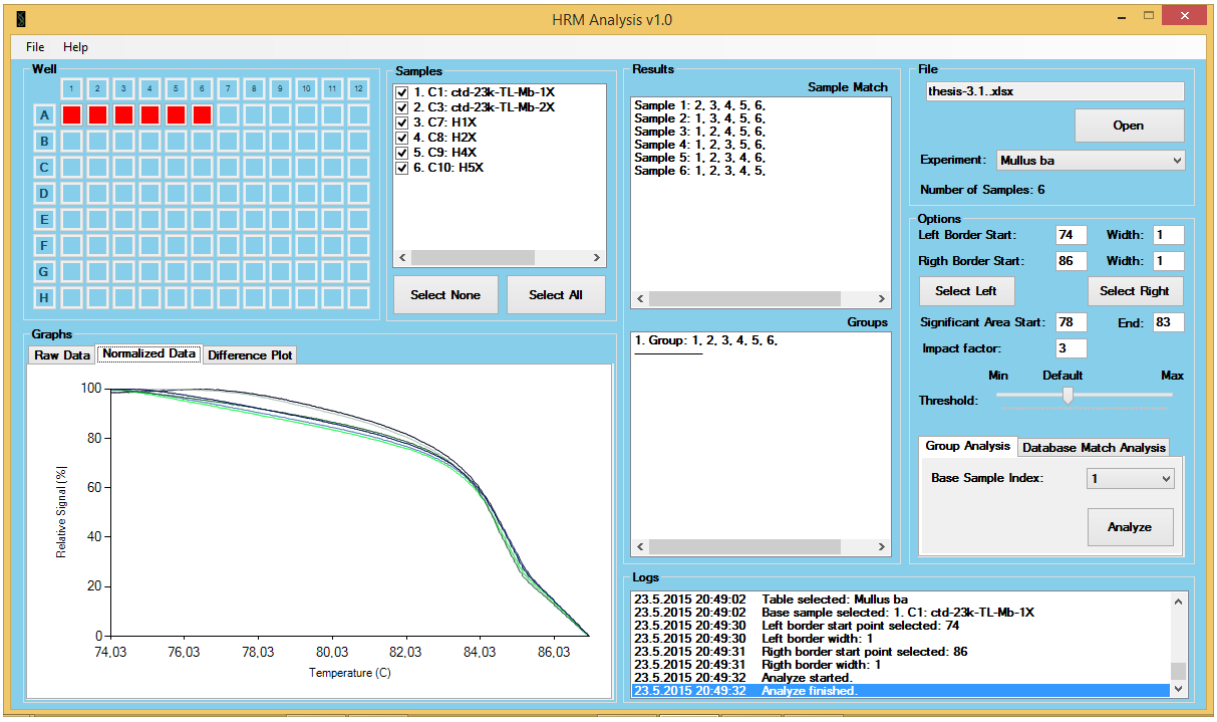

Figure G.20. Normalization and grouping outputs of Mullus barbatus organisms from HRM software

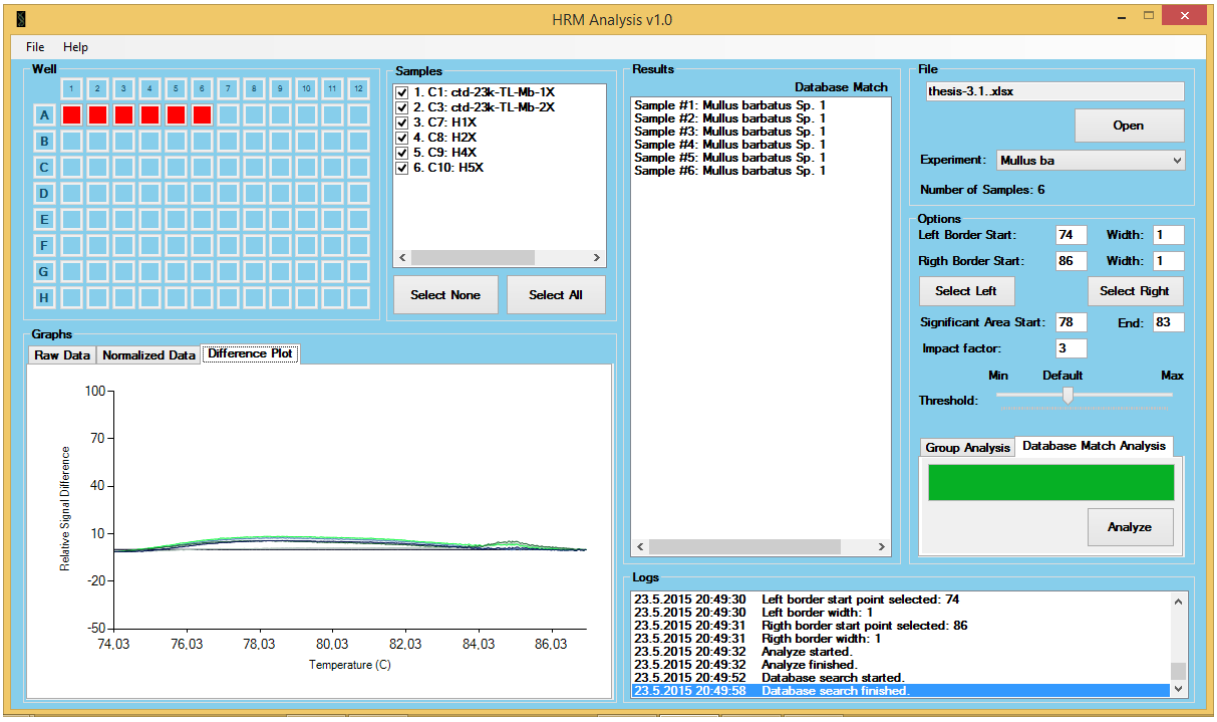

Figure G.21. Difference plot and database match analysis outputs of Mullus barbatus organisms from HRM software

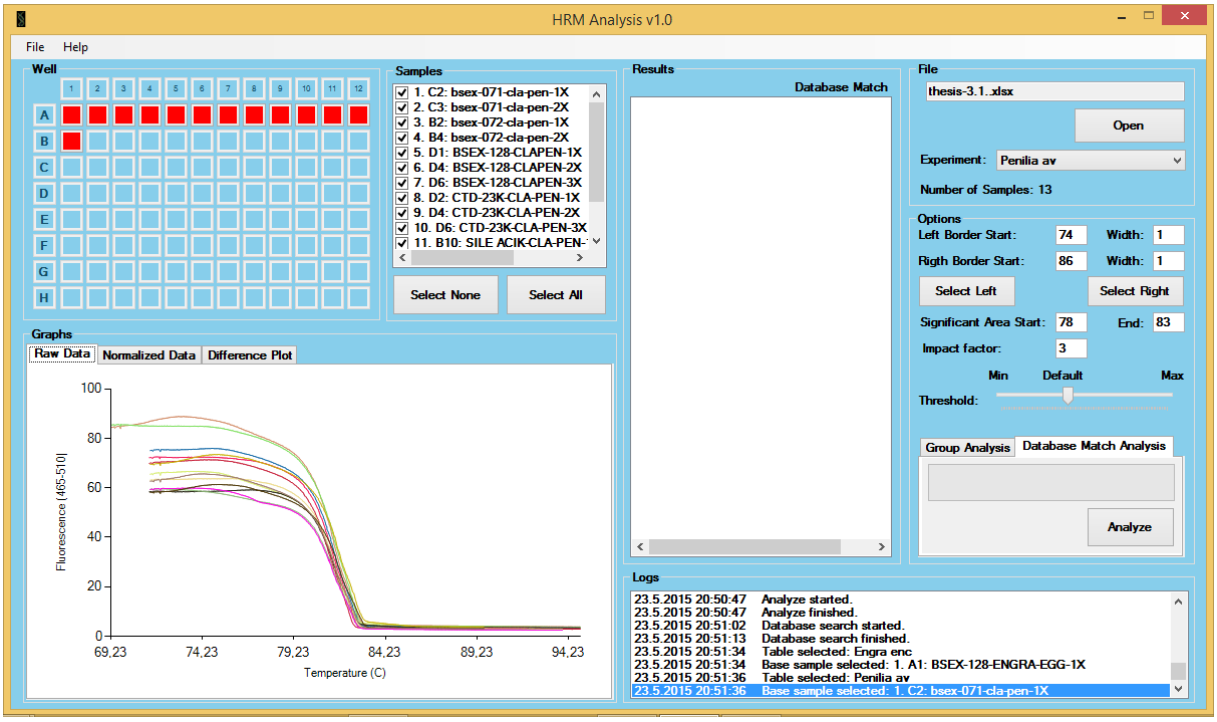

Figure G.22. Raw Melt curve data outputs of Penilia avirostris organisms from HRM software

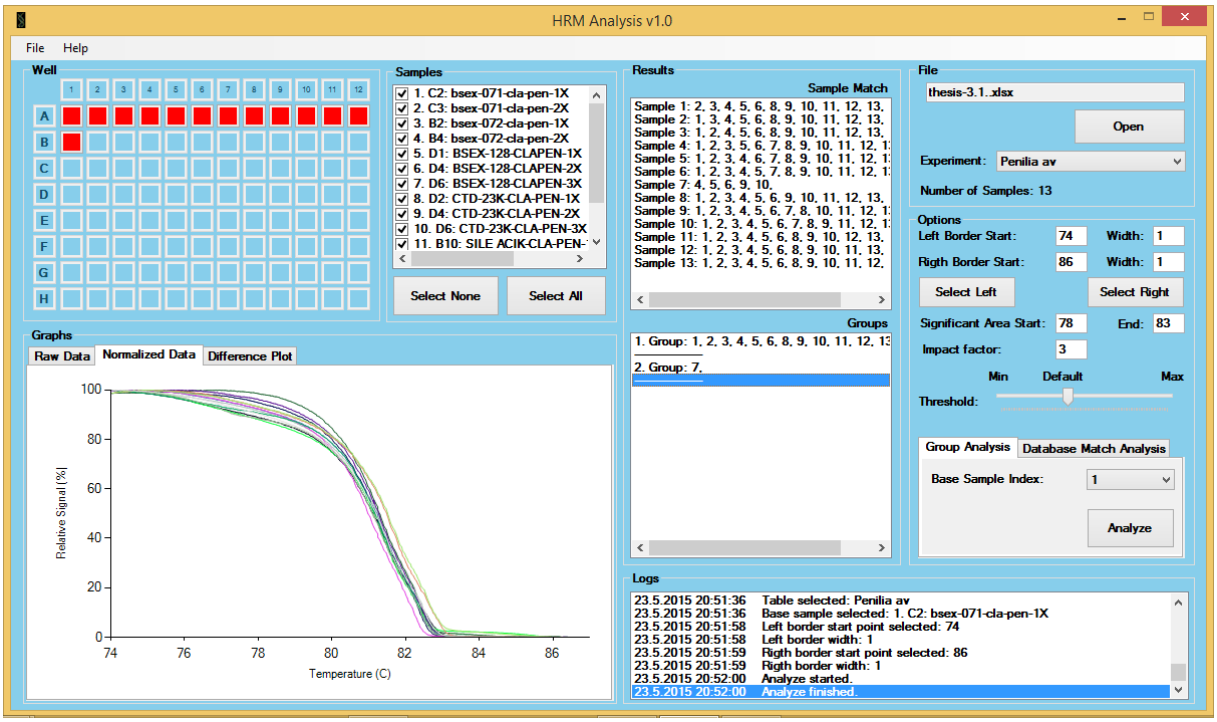

Figure G.23. Normalization and grouping outputs of Penilia avirostris organisms from HRM software

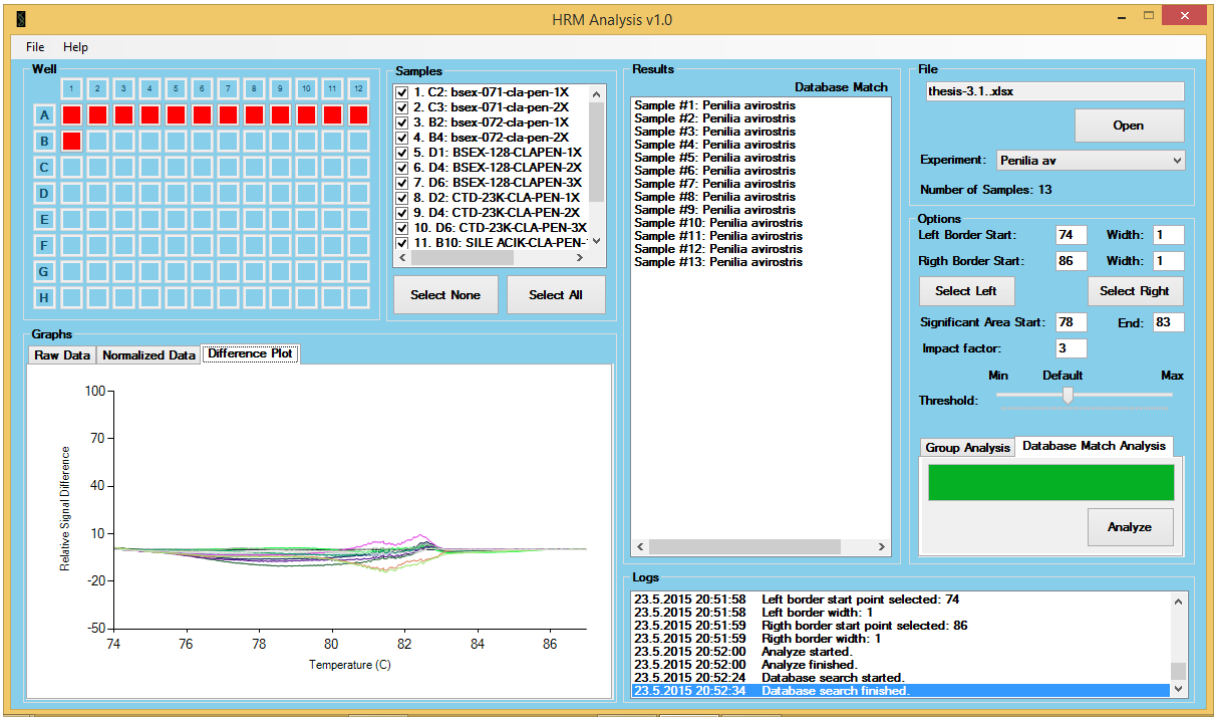

Figure G.24. Difference plot and database match analysis outputs of Penilia avirostris organisms from HRM software

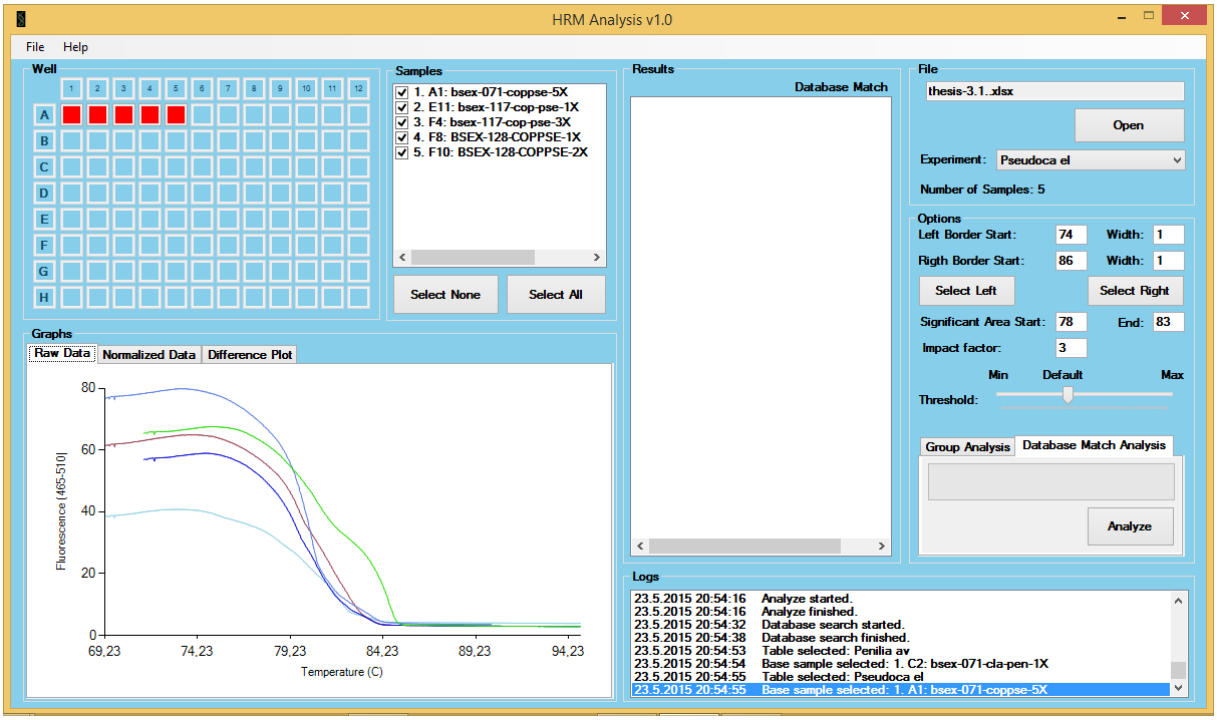

Figure G.25. Raw Melt curve data outputs of Pseudocalanus elongatus organisms from HRM software

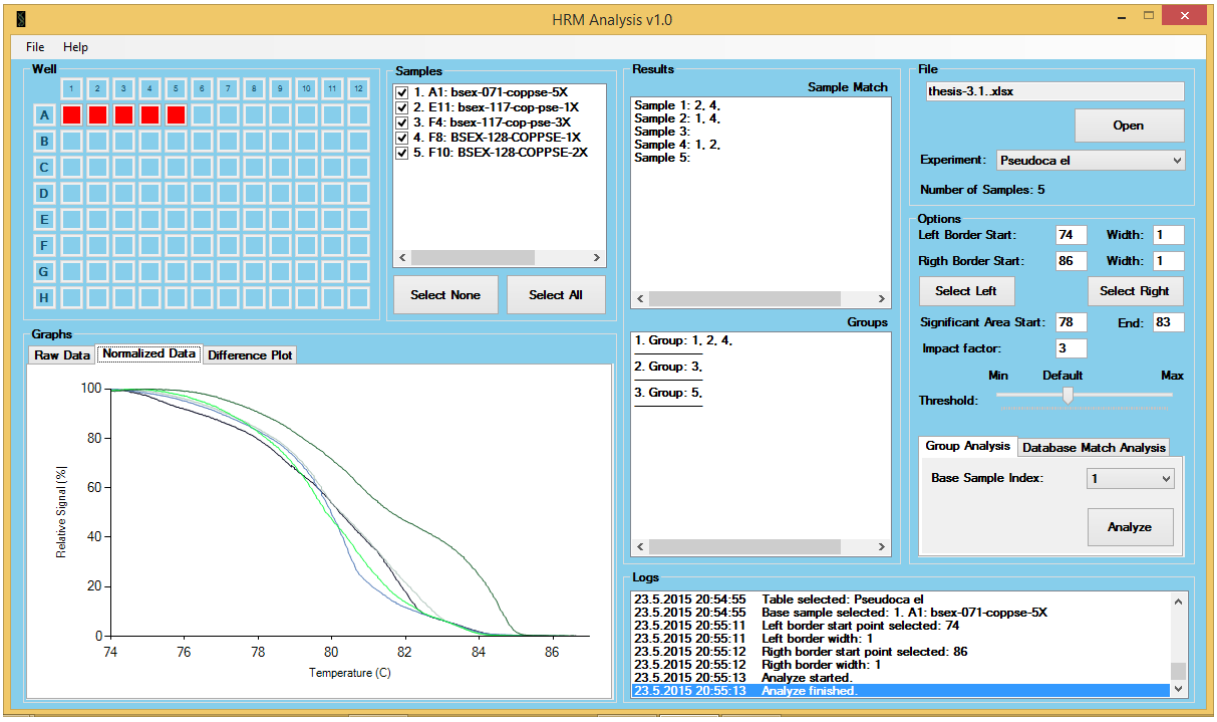

Figure G.26. Normalization and grouping outputs of Pseudocalanus elongatus organisms from HRM software

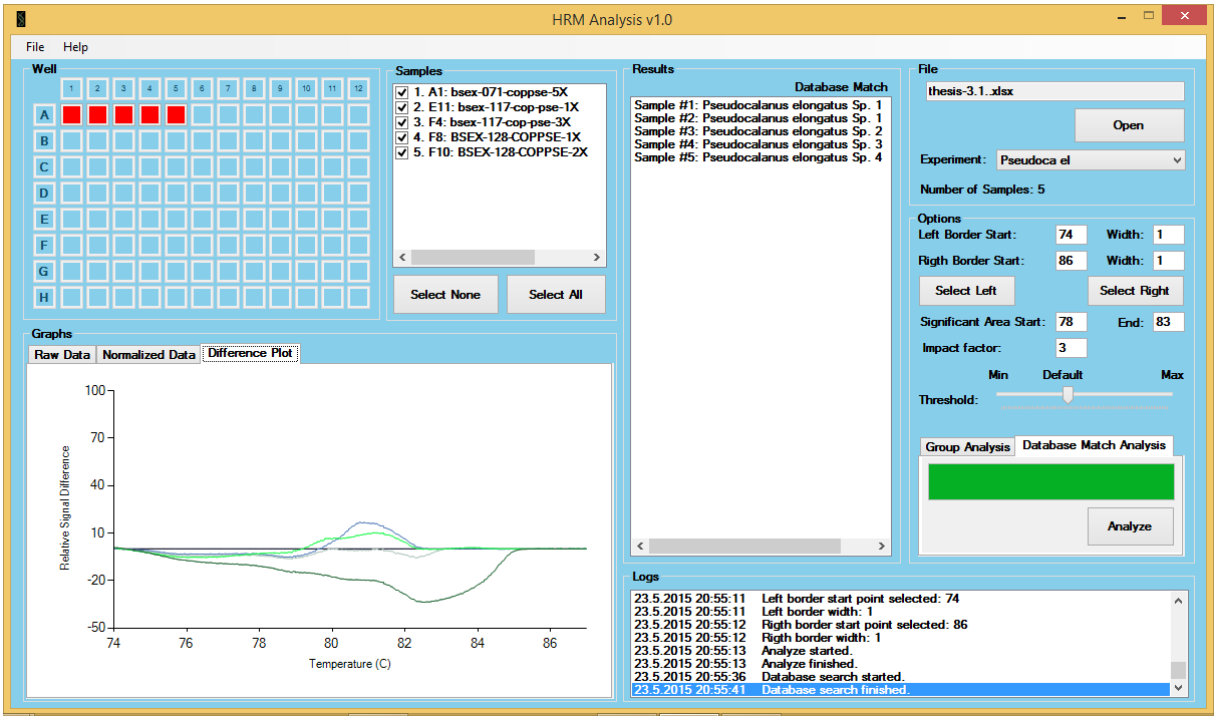

Figure G.27. Difference plot and database match analysis outputs of Pseudocalanus elongatus organisms from HRM software

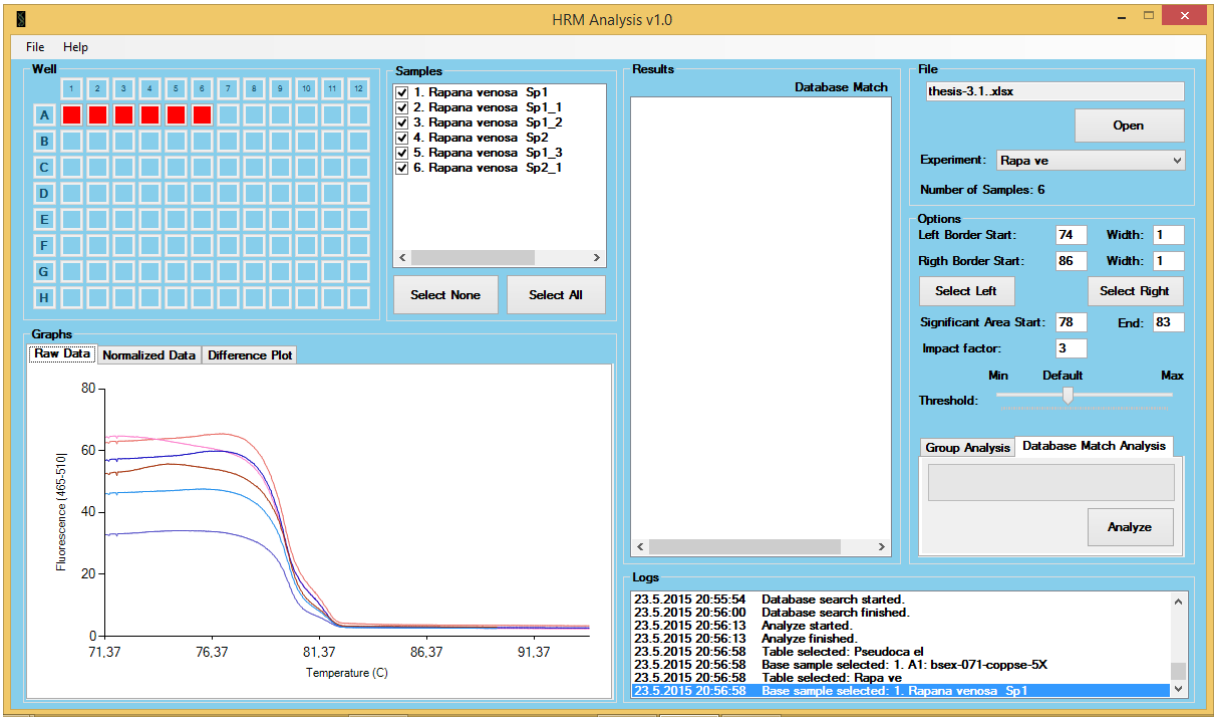

Figure G.28. Raw Melt curve data outputs of Rapana venosa organisms from HRM software

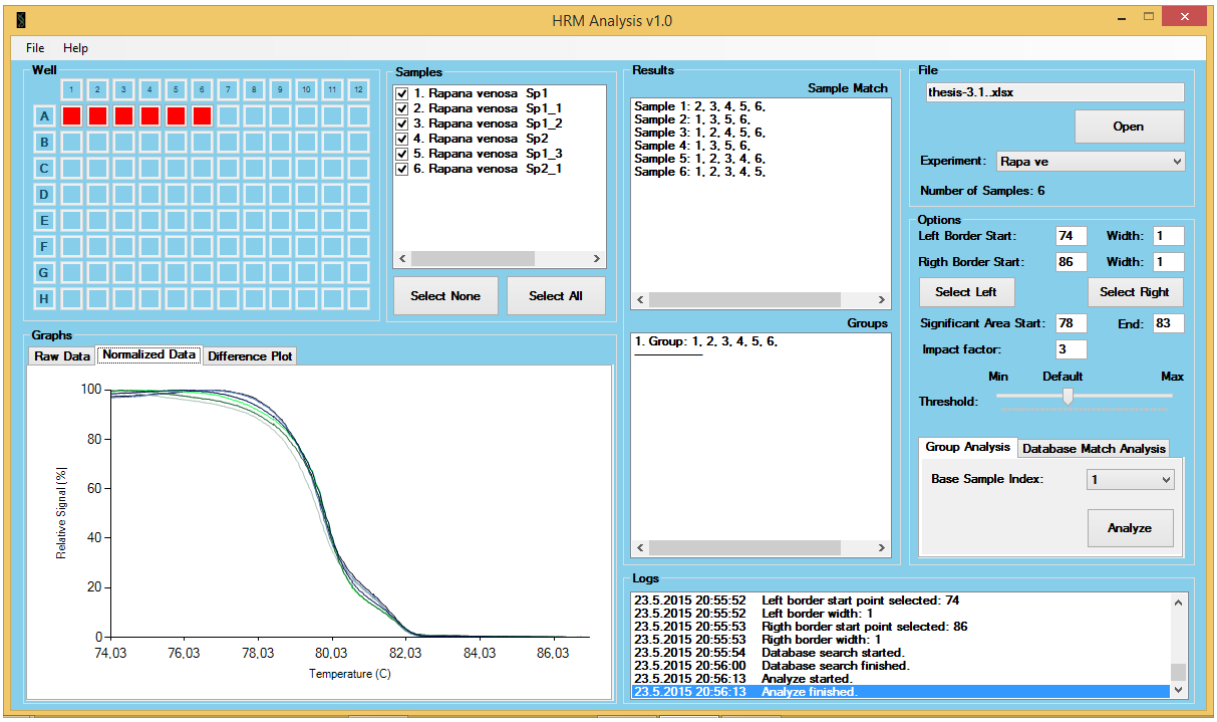

Figure G.29 Normalization and grouping outputs of Rapana venosa organisms from HRM software

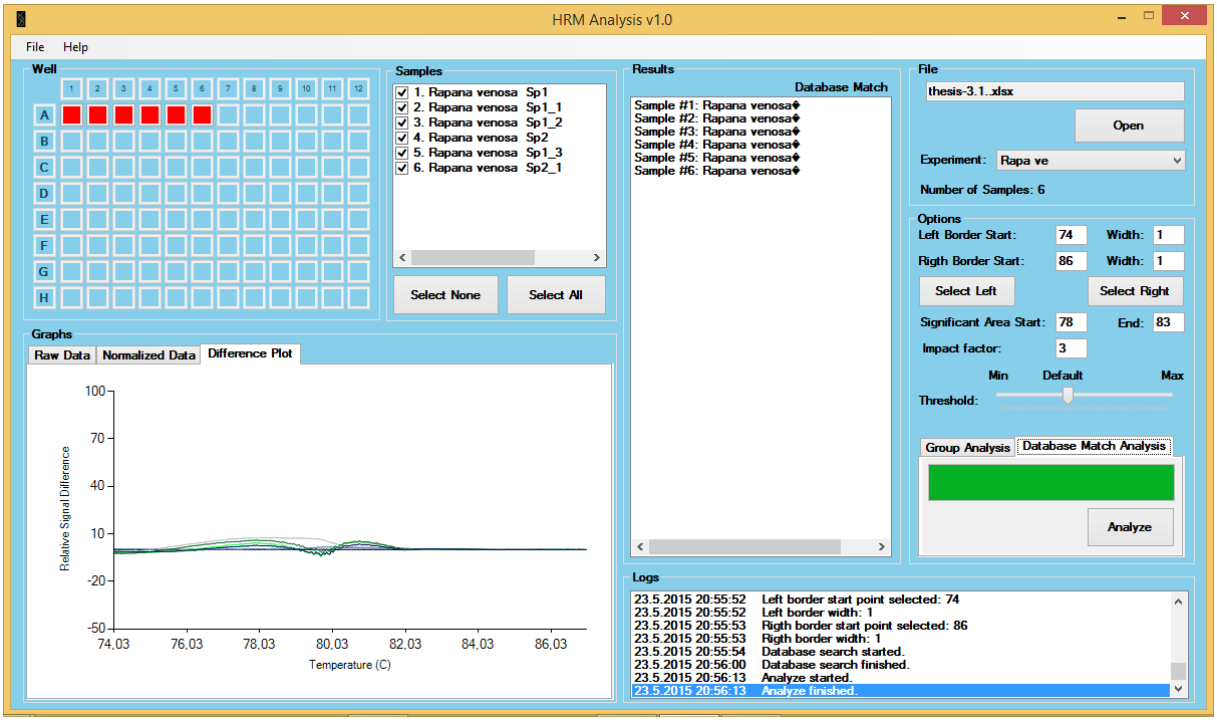

Figure G.30. Difference plot and database match analysis outputs of Rapana venosa organisms from HRM software