PROBING THE MOLECULAR DIVERSITY OF *SYMBIODINIUM* SPECIES ASSOCIATED WITH CORALS IN THE INTERTIDAL REEF HABITAT

Dissertation submitted in partial fulfillment of for the requirement of the degree of

MASTER OF TECHNOLOGY

IN

BIOTECHNOLOGY

By

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TABLE OF CONTENTS

CANDIDATE'S DECLARATION

I hereby declare that work presented in this report entitled **"Probing the molecular diversity of Symbiodinium species associated with corals in the intertidal reef habitat"** in partial fulfilment of the requirement for the award of degree in Master of Technology in the Department of Biotechnology from Thapar institute of Engineering and Technology, Patiala, Punjab, is an authentic record of my own work carried out under the supervision of Dr. Manikandan B, Senior Scientist at Council of Scientific and Industrial Research – National Institute of Oceanography, Goa.

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This is to certify that the above statement made is true to best of my knowledge.

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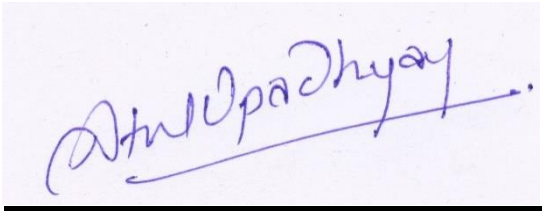
CERTIFICATE

This is to certify that the dissertation "Probing the molecular diversity of Symbiodinium species associated with corals in the intertidal reefs habitat" submitted by Ms Soumi Biswas is in partial fulfilment of the requirement for the award of degree in Master of Technology in the Department of Biotechnology from Thapar institute of Engineering and Technology, Patiala, Punjab, is a bonafide work carried out under my supervision and guidance.

To the best of my knowledge, the dissertation work contains no materials previously submitted to any other university or institute for the purpose of receiving a degree or certificate.



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ABSTRACT

Coral is a type of marine invertebrate animal that belongs to the phylum Cnidaria. They are sometimes referred to as "coral polyps" and are classified into two major groups: hard corals and soft corals. They are found in warm, shallow waters, particularly in tropical regions.

Coral reefs are large underwater structures that are formed by colonies of coral polyps. These reefs are considered to be one of the most diverse and productive ecosystems on Earth, providing a habitat for a vast array of marine life. Coral reefs are typically found in clear, warm waters with little to no pollution and a high amount of sunlight.

Coral reefs are known for their vibrant and colorful appearance, with corals often displaying a variety of shapes, sizes, and colors. They play a crucial role in providing food, shelter, and protection for numerous marine species, including fish, crustaceans, and mollusks. Coral reefs also act as natural barriers, protecting coastlines from erosion and storm damage.

While coral reefs are incredibly important, they are also facing numerous threats. Climate change, pollution, overfishing, and destructive fishing practices, such as dynamite fishing and coral mining, are all contributing to the decline of coral reefs across the globe. When coral reefs suffer, the delicate balance of the marine ecosystem is disturbed, leading to a loss of biodiversity and significant environmental consequences.

Efforts are being made to protect and conserve coral reefs through initiatives such as marine protected areas, sustainable tourism practices, and coral gardening. These measures aim to mitigate the effects of human activities on coral reefs and promote their recovery and preservation for future generations.

CHAPTER 1: INTRODUCTION

1.1 Coral:

Corals are marine invertebrates within the class Anthozoa of the phylum Cnidaria. They typically form compact colonies of many identical individual polyps. Corals are related to sea anemones, and they all share the same simple structure, the polyp. The polyp resembles a tin can that is open at only one end. At this open end, there is a mouth encircled by a ring of tentacles. The coral polyp utilizes its tentacles, which contain stinging cells known as nematocysts, to capture small organisms that come too close while swimming. Corals have digestive and reproductive tissues within their polyp bodies. What sets them apart from sea anemones is their ability to produce a mineral skeleton.

Shallow water corals residing in warm environments often have an additional source of food provided by the zooxanthellae. Single-celled algae engage in photosynthesis and share some of the food they produce from the sun's energy with their hosts. In return, the coral animal provides nutrients to the algae. It is this relationship that allows shallow water corals to grow fast enough to build the enormous structures we call reefs. The Zooxanthellae also contribute significantly to the green, brown, and reddish colors observed in corals. The less common purple, blue, and mauve colours found in some corals the coral makes itself.

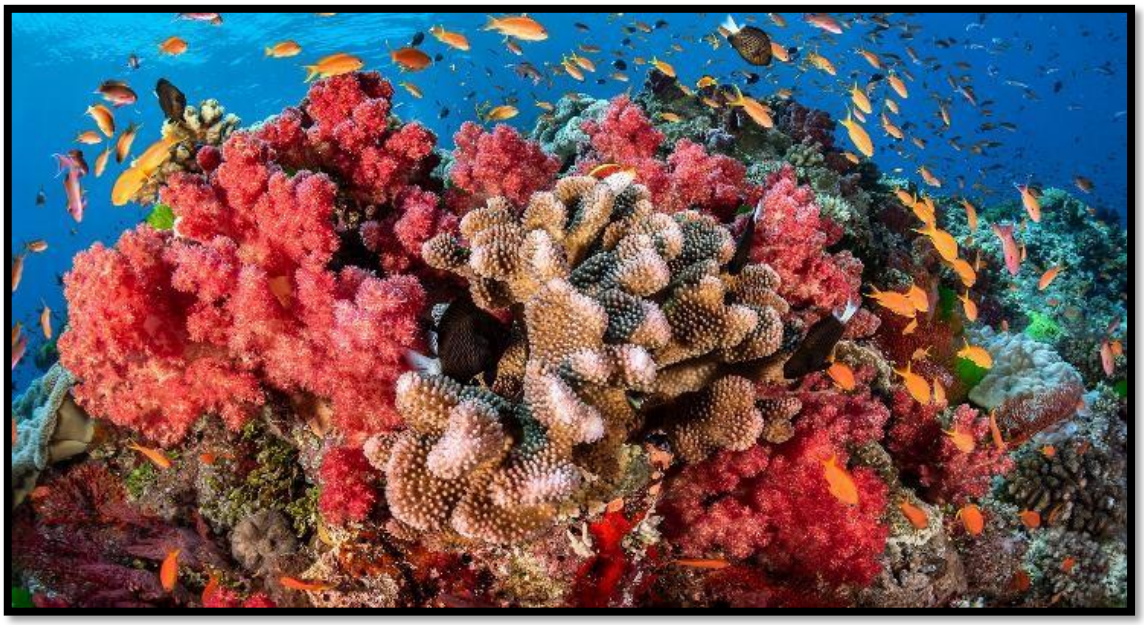


Figure 1: A structure of a coral

1.2 Coral reef:

A coral reef is an underwater ecosystem characterized by reef-building corals. Coral reefs protect the coastlines from storms and erosion, and are a source of food and new medicines. They are colourful because of the algae that grows on them. They are made of thin layers of calcium carbonate. They are animals, not pants and are made up of colonies of hundreds to thousands of tiny individual corals called polyps. Coral polyps are tiny, soft-bodied organisms related to sea anemones and jelly fish. They have special adaptations for defence, or protection from predators and other natural stresses formed in the coral reef environment. At the base of corals, there is a sturdy and protective limestone skeleton known as a calicle, which plays a crucial role in forming the structure of coral reefs. Reefs start forming when a polyp anchors itself to a rock on the seabed, and subsequently, it divides or buds into thousands of clones. They are formed of colonies of coral polyps held together by calcium carbonate.

Coral reefs are diverse and productive but sensitive ecosystems. Due to the impact of climate change, these organisms are in danger of dying out, mainly through the process of coral bleaching, which is the process by which zooxanthellae (algal endosymbionts) are expelled from their respective coral hosts, causing the coral to lose colour and become white. Coral bleaching has been linked to increases in sea surface temperatures as well as an increase in light intensity. We reviewed the different zooxanthellae taxa and their ecological traits, as well as the information available on the protective mechanisms present in zooxanthellae cells when they experience environmental stress conditions, such as temperature fluctuations, specifically concentrating on heat shock proteins and their response to antioxidant stress. The eight clades (A–H) previously recognised were reorganised into seven existing genera. Different zooxanthellae taxa exhibit different ecological traits such as their photosynthetic stress responses to light and temperature. Zooxanthellae have the ability to regulate the number and

type of heat shock proteins (Hsps) they produce during a heat response. They can also regulate the host's respective Hsps. Antioxidant responses that can prevent coral hosts from expelling the zooxanthellae, can be found both within exposed coral tissue and the zooxanthellae cells.

Figure 2: A colony of coral reef found in aquatic environment

1.2.1 Values of coral reef:

Coral reefs are often called the "rainforests of the sea" because of the diversity of life found in the habitats created by corals, It is termed as the coral reefs diversity and about 25% of the ocean's fish depend on healthy coral reefs. Fishes and other organisms shelter, find food, reproduce, and rear their young in the many nooks and crannies formed by corals. are very complex ecosystems that provide valuable habitat for fish and other animals with their beautiful and unique structures. These structures provide shelter for many organisms such as fish, marine worms, clams and many other animals and plants that all play a vital role in the coral reef ecosystem. They also protect coastlines from storms and erosion, provide jobs for local communities, and offer opportunities for recreation. The enormous diversity of coral reef

organisms also provides potential for new medicines or other products that may be developed from biochemicals that these organisms produce. Most coral reef organisms have not been studied for their potential benefits to medicine and industry.

1.3 Coral bleaching:

Coral bleaching can be described as the process by which zooxanthellae (algal symbionts) are expelled from the gastrodermal cavity (tissue) of the respective coral host. Thereafter coral whitening occurs, displaying the CaCO3 skeletal structure of the coral which causes stress to the coral and it can then die. Most coral bleaching events are due to an increase in temperature above the normal stable temperature in which the holobiont survives. Scleractinian corals are reef-building corals that live in a mutualistic symbiotic relationship with single-celled zooxanthellae, referred to as dinoflagellates, belonging to the genus Symbiodinium. The specific clade to which these resident Symbiodinium cells belong, can be linked to their host's susceptibility to variation in oceanic temperatures, thus variation in thermal tolerance is observed among individual colonies and host species. Expulsion of zooxanthellae from the coral's internal tissues during coral bleaching is regulated by the rate of photoinhibition and photo-damage to the zooxanthellae's thylakoid membranes' integrity and fluidity. These regulating factors are also influenced by the respective zooxanthellae clade/genus as well as the scleractinian coral host.

Coral bleaching is directly linked to elevated sea surface temperatures (SSTs) as well as an increase in light intensity. The elevated SSTs may result in an increased amount of reactive oxygen species (ROS) produced by the symbiont, which concurrently results in oxidative stress in both coral host and symbiont. During oxidative stress, ROS are produced in the endosymbiont's chloroplast, further producing hydrogen peroxide (H2O2). H2O2 can thereafter diffuse into the coral's cytoplasm and cause oxidative damage to the coral tissue.

Damage is not only caused in the host's tissue but also within the thylakoid membranes of the endosymbiont.

Figure 3: A fully bleached coral

1.4 Coral reef locations:

Coral reefs are present in the areas of Gulf of Kutch, Gulf of Mannar, Andaman & Nicobar, Lakshadweep Islands and Malvan.

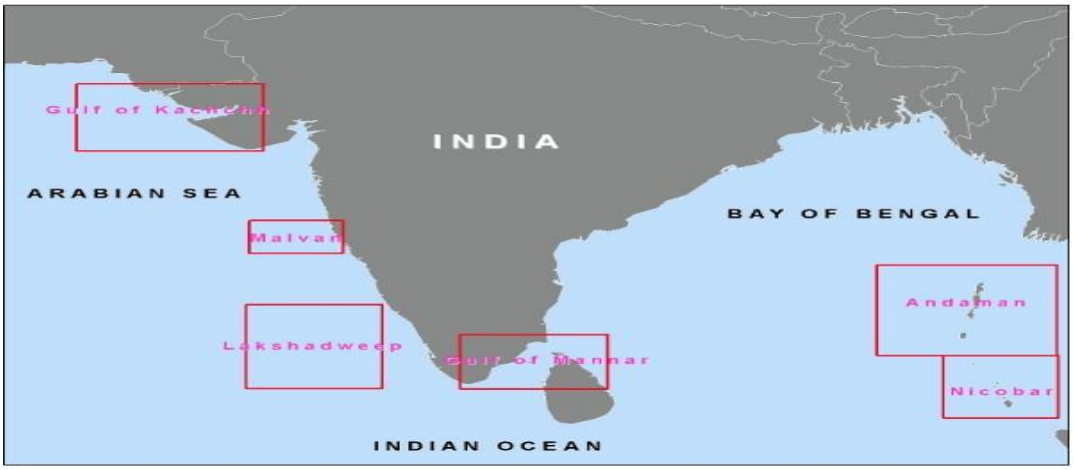


Figure 4: Distribution of corals along the Indian Coast

1.5 Major threats for the corals:

a) Natural: Environmental-Temperature, Sediment Deposition, Salinity, pH, etc

b) Anthropogenic: Mining, Bottom Fishing, Tourism, pollution, etc.

c) Conservation threats: Ocean acidification, fishing, coral bleaching, sunscreen, and water quality.

1.6 Environmental conditions suitable for coral:

a) Sunlight: Corals need to grow in shallow water where sunlight can reach them. Corals depend on the zooxanthellae (algae) that grow inside of them for oxygen and other things, and since these algae needs sunlight to survive, corals also need sunlight to survive. Corals rarely develop in water deeper than 165 feet (50 meters).

b) Clear water: Corals are sensitive to pollution and sediments. Sediment can create cloudy water and be deposited on corals, blocking out the sun and harming the polyps. Most reef-building corals depend upon zooxanthellae (tiny little algae that grow inside of them) to photosynthesize and provide food. If the water becomes cloudy or murky, or if corals are covered in sediment, the sunlight can't get to the zooxanthellae and the corals lose that important food source.

c) Warm water: Though it varies largely on geography and the species of coral, many reef-building corals have a narrow temperature range in which they can thrive. Most hard corals prefer water temperatures that range between 73° and 84° Fahrenheit (23° and 29° Celsius), though some can tolerate temperatures as low as 68° F (20° C) and as high as 90° F (32° C).

d) Saltwater: Corals need saltwater to survive and require a certain balance in the ratio of salt to water.

1.7 Common coral species and it's identification:

a) Favites is a genus of brain coral and is classified as a large polyp stony coral. It is a species-rich genus with a wide distribution in the Indo-Pacific region from the Red Sea to Japan and Fiji. There are about 20 different species. They usually have smaller polyps that share a common wall with adjacent polyps, although those with a clear separation also exist. Differentiation from other brain corals such as Favia corals or Goniastrea corals is therefore

difficult, especially on live animals. These corals have low maintenance requirements for lighting, food and equipment and are therefore very suitable for beginners as a first stony coral when stocking the tank. They also cope well with higher nutrient levels. They like medium to low light and medium flow.
(IUCN status - Near threatened).

b) Goniopora is a genus of stony corals from the family Poritidae. They belong to the large-polyp stony corals, although they are actually closely related to the small-polyp stony corals. This relationship is most evident in their skeletal structure. Goniopora, however, have conspicuously long, fleshy polyps that can reach up to 10 cm in length, depending on the species. Because of their shape they are also called margarite corals or in English flowerpot corals. They can fully retract these tentacles into their skeleton when disturbed. In shape they are similar to Alveopora, the difference between Alveopora and Goniopora is that Goniopora have tentacles with 24 tips, while Alveopora have only 12 tentacle tips. Thus, the distinction between Goniopora and Alveopora can be made simply by the number of tentacles. Especially for the species with small polyps the differentiation to Porites and similar species can also be a problem. The species with the smallest polyps, Goniopora stutchburyi, which is commonly called "carpet of love", has meanwhile been classified in its own genus Bernardpora. As a special feature, Goniopora can self-reproduce via offshoots, a process known in science as "fragging," effectively asexually forming their own frags. Most often Goniopora are green or beige in color, less common are yellow-green animals, occasionally blue-purple and very rarely red color forms. Multicolored Goniopora are especially sought after, like the Golden Inferno Goniopora, which is multicolored red-yellow. Red-blue or green-blue color forms also occur from time to time, and Bernardpora has spectacular orange-based multicolored Rainbow color forms.
(IUCN status – Near threatened).

c) Porites is a genus of stony corals with a circumtropical distribution. They are classified as small polyp stony corals. They tend to form encrusting, columnar or helmet-shaped colonies that can reach a tremendous age and a considerable size of several meters in diameter. A few species also grow branched and branch-like. If they reach the surface of the water, the upper portion dies off and only the sides continue to grow, allowing erosion to form a microatoll. Porites are zooxanthellate and feed on light and dissolved

nutrients. They require very clean water and high light levels for optimal coloration. Porites are mostly beige to brown in color, rarely green, purple-bluish or striking yellow, and very rarely red specimens are said to occur. (IUCN status – Least concern).

d) Platygyra is a genus of stony corals and belongs to the Merulinidae. The growth form is similar to brain corals - colonies are plate-shaped to massive. Most conspicuous are the elongated valleys of polyp walls that encompass several polyps with multiple mouths. The taxonomic situation, as with many corals, is a disaster - differentiation from related species such as Favites or Goniastrea is rarely cleanly possible, and if so then usually only microscopically on dead specimens of the skeleton or by DNA analysis. Revisions are also common. We therefore simply use the name for all brain coral-like LPS plates with the typical polyp valleys. The requirements are fortunately also similar across species - medium current, medium to weak lighting, nutrients may also be higher - in short: typical for LPS. Most interesting are the different color variations with contrasting colors on the "valleys" and "ridges", often "monochromatic" brown with green/purple, but sometimes both multicolored. In addition, patterned specimens with thin transverse stripes appear especially on the combs.

(IUCN status – Least concern).

e) Symphyllia was a genus of large polyp stony corals (LPS). They occur in the Red Sea, the tropical Indo-Pacific and from Japan to the South Sea Islands of the Pacific. They preferentially colonize reef slopes and surf-protected marginal reefs. Typically, colonies grow in a massive dome shape, the corallites are flabello-maeandroid, or twisted like a brain, and consist of a valley with ridges as a fringe, which are often contrastingly colored. Each corallite houses several polyps, as seen by the many mouth openings. In the meantime, the genus Symphyllia no longer exists, as after taxonomic revisions most species have been reassigned, most to Lobophyllia. However, it is still needed for CITES purposes, which is why we as dealers can't get away from it for now. Distinguishing "Symphllia" from Lobophyllia has often been difficult; in general, species designated as Symphyllia tend to have larger, more branched corallites with more mouth openings than Lobophyllia, whose corallites are often more separated and do not exhibit ridges dividing the interior surface. Known species include Symphyllia (Lobophyllia) valenciennesi or Symphyllia (Australophyllia) willsoni. During the day Symphyllia hide their tentacles and mainly perform

photosynthesis with their zooxanthellae, while at night they extend their tentacles and also go out to catch plankton. They are robust and tolerate even high nutrient levels, and require only weak lighting, otherwise they bleach out easily, especially animals from muddy habitats with strong turbidity.
(IUCN status – Least concern).

f) Turbinaria is a genus of large polyp stony corals and belong to the family Dendrophylliadae, together with e.g. Tubastrea or Duncanopsammia. They are found in the Red Sea, Indian Ocean, Japan and the southern Pacific. Usually the skeletons grow in lettuce leaf like pagodas, the shape is often mushroom or chalice shaped, the polyps are often deeply embedded in the skeleton. It is a zooxanthellate coral that feeds on light with its symbiotic algae. Like many LPS, it displays its polyps at night to catch plankton and can be fed powdered or frozen foods. They are hardy and do well with both higher nutrient levels and weaker lighting, making them attractive to beginners. Due to their shape, you will need a strong enough current to prevent sediment buildup and tissue damage. Commonly available species are Turbinaria reniformis with small polyps in yellow to green and brown or purple tissue, or Turbinaria peltata, mostly turquoise green, with larger polyps with clearly visible tentacles.
(IUCN status – Vulnerable).

CHAPTER 2: *SYMBIODINIUM* CELLS

2.1 Genus *Symbiodinium*:

Corals are particularly susceptible to elevated seawater temperatures and exhibit bleaching or lose their algal endosymbionts of the genus *Symbiodinium* under stress. *Symbiodinium* belong to phylum Dinoflagellata, which is one of the largest groups of marine phytoplankton. *Symbiodinium* cells perform photosynthesis within the coral host cells. The photosynthetically-produced organic nutrients are translocated to the coral host and contribute substantially to its growth and calcification. In return, the coral host provides a protective environment and a supply of nutritious metabolic waste products to the symbionts. Such partnership between the coral host and its symbionts is known to be highly sensitive to environmental stressors such as high seawater temperature and high solar irradiance.

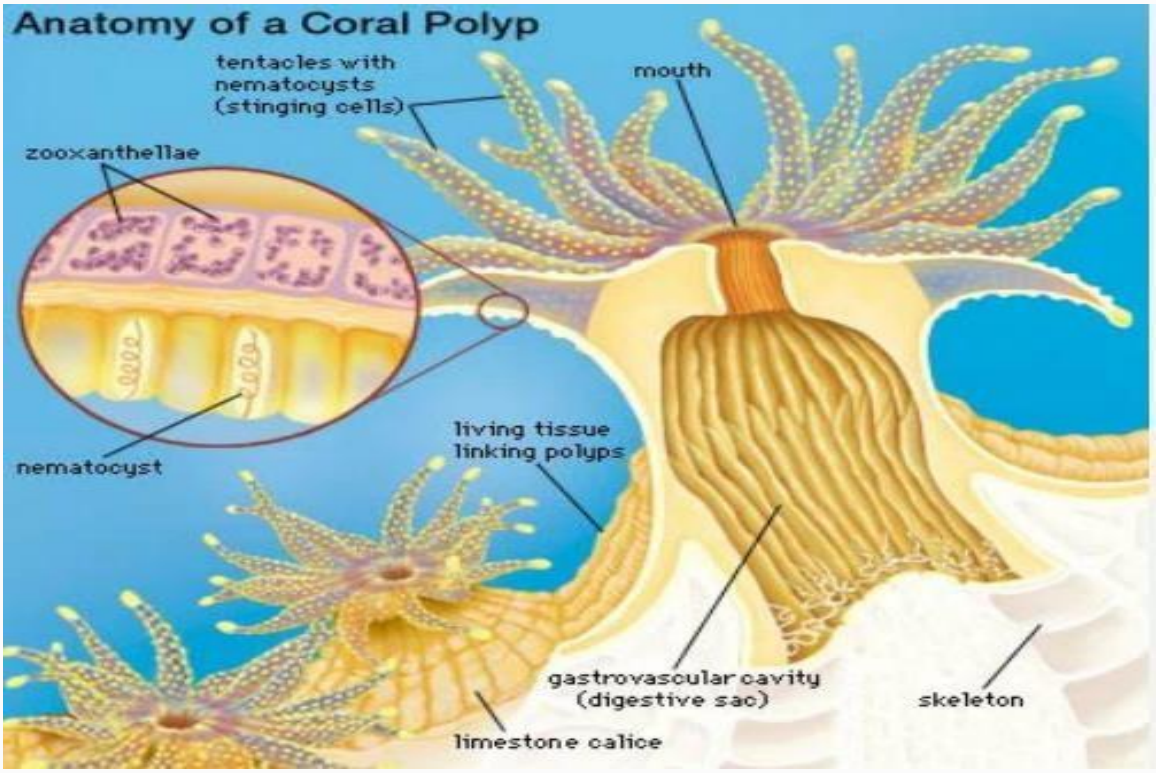


Figure 11: Anatomy of a coral polyp

CHAPTER 3: SYMBIOTIC RELATIONSHIP BETWEEN CORALS AND SYMBIODINIUM

The mutually beneficial relationship between algae and modern corals — which provides algae with shelter, gives coral reefs their colours and supplies both organisms with nutrients Algae belonging to the group known as dinoflagellates live inside the corals' tissues. The algae use photosynthesis to produce nutrients, many of which they pass to the corals' cells. The corals in turn emit waste products in the form of ammonium, which the algae consume as a nutrient. This relationship keeps the nutrients recycling within the coral rather than drifting away in ocean currents and can greatly increase the coral's food supply. Symbiosis also helps build reefs — corals that host algae can deposit calcium carbonate, the hard skeleton that forms the reefs, up to 10 times faster than non-symbiotic corals.

Zooxanthellae is the brown-yellow algae that lives in coral's gastrodermis, and is the common name of the broader *Symbiodinium* genus. Zooxanthellae is a term for any dinoflagellate that participates in symbiosis with sponges, coral, clams, mollusks, flatworms, jellyfish, etc. It is an algal protist that is best known for its symbiotic relationship with marine coral. Zooxanthellae usually occur in extremely high densities on their host, enhancing the constant exchange of nutrients between them and their host.

Corals are usually colonies of polyps. Polyps are live coral tissue extensions that cover the calcium carbonate structure, and are usually only a few millimeters thick. The tissue has two layers, the epidermis and the gastrodermis, where the zooxanthellae live. Zooxanthellae are unicellular and spherical with two flagella that fall off once they are incorporated within a host. Zooxanthellae undergo asexual reproduction by division, and most of their energy comes from performing photosynthesis using the byproducts of cellular respiration produced from the host coral.

The symbiotic relationship between zooxanthellae and marine coral is understood on a basic level. The coral polyps do cellular respiration, thus producing carbon dioxide and water as byproducts. The zooxanthellae then take up these byproducts to carry out photosynthesis. The products of photosynthesis include sugars, lipids, and oxygen, which the coral polyps thus uptake for growth and cellular respiration, and the cycle continues. The photosynthesis byproducts are more specifically used to make proteins and carbohydrates in order to produce calcium carbonate for the coral to grow. Furthermore, the oxygen is used by the coral to help remove wastes. This recycling of nutrients in between these symbionts is extremely efficient, resulting in the ability to live in nutrient poor waters. About ninety percent of the material produced by photosynthesis is thought to be used by the coral. In terms of disease, the zooxanthellae is commonly the point of attack, rather than the coral itself. For example, the Montastrae species, which causes Yellow Band Disease, affects the zooxanthellae directly rather than the coral.

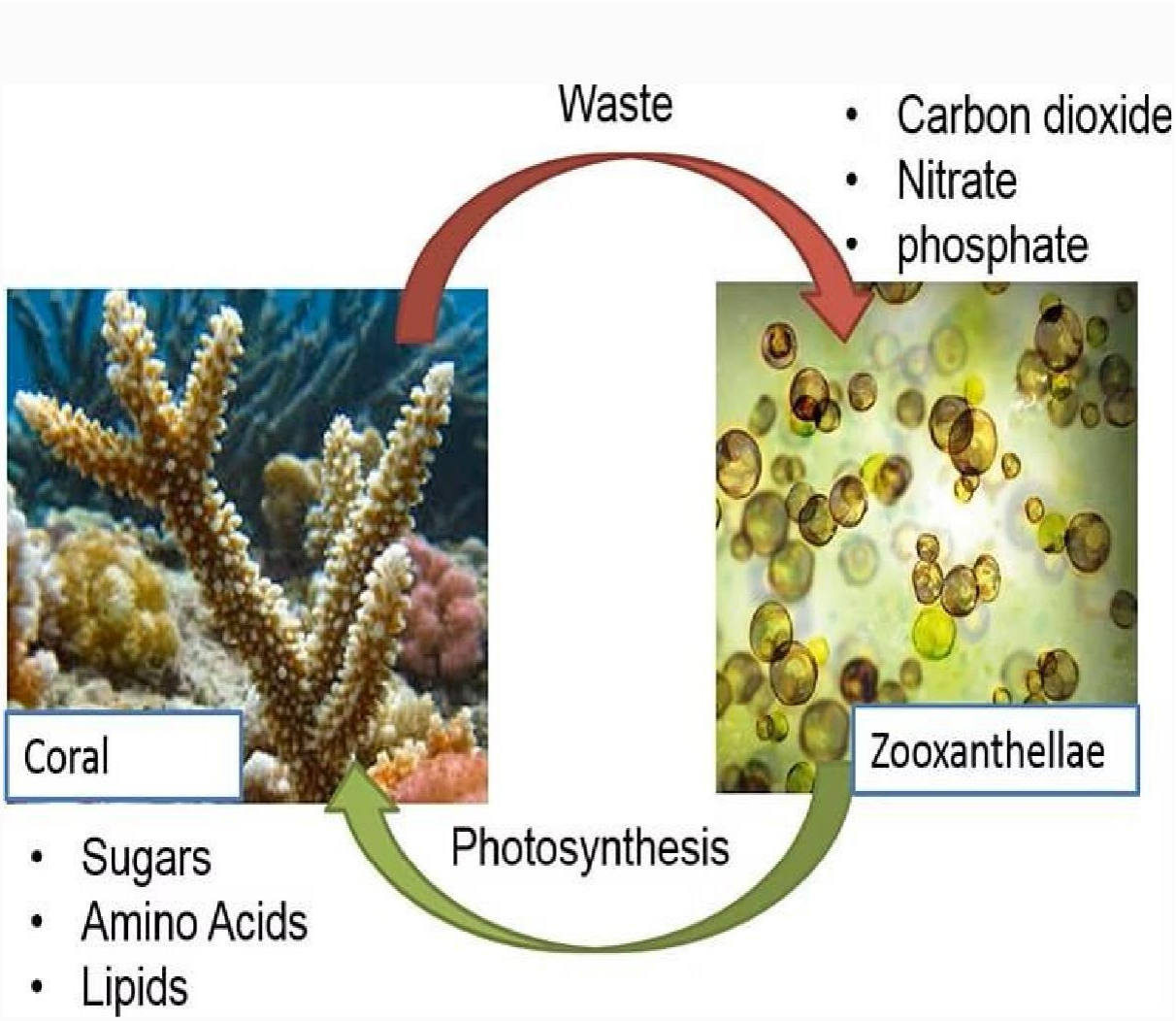


Figure 12: Cycle representing the mutualistic relationship between coral and the algal host

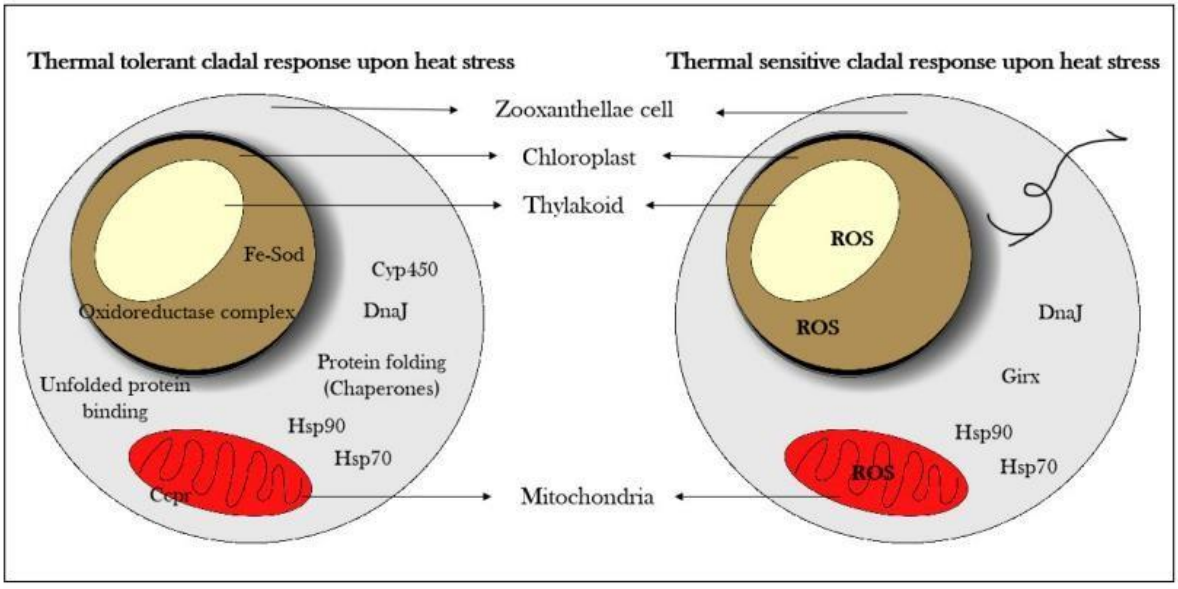


Figure 13: Protective mechanisms that are induced to actively counteract heat/thermal stress within both sensitive and tolerant zooxanthellae species

CHAPTER 4: THE GULF OF KACHCHH

Gulf of Kachchhh is a shallow water body, with depth extending from 20 m at the head of the gulf to 60 m at the mouth, while the average depth is 30 m, the minimum is 3 m. The high tidal influx covers the low-lying area of about 1500 km^2 comprising networks of creeks and alluvial marshy tidal flats in the interior region. The region experiences 4-6m of high tide and low tide, which makes it unique out of the other marine biodiversity location. The creek system consists of three main creeks namely Nakti, Kandla and Hansthal and little Gulf of Kutch interconnecting through many other big and small creeks. All along the coast, very few rivers drain into the Gulf of Kutch and they carry only a small quantity of freshwater, except during brief monsoon. The southern shore of the Gulf has abundant islands and inlets covered with mangroves and surrounded by coral reefs. The northern shore is mostly sandy or muddy provoked by several fish flocks. The residence or turnover time of the gulf water ranges from 8-51 days, decreasing upstream.

Figure 14: Location of Gulf of Kachchh in India

4.1 Physiological condition:

The gulf is surrounded by arid to semi-arid landmass. The relative humidity is the highest in August is 82% and the lowest in December-January is 60%. Atmospheric temperature varies from 10°C in January to 35°C in May-June. River runoff is negligible, as the streams which drain into the gulf carry only a small quantity of freshwater except during the deep spell of monsoon in July-September and have dams across most of them converting the gulf to a negative water balance body. Cyclonic storms periodically strike North Gujarat, particularly

the Kutch and Saurashtra regions. Cyclones produce large tidal waves, inundating coastal stretches temporarily and also cause appreciable damage to coastal or near shore structures. Tides in the gulf are mixed, predominantly semi-diurnal type with a large diurnal inequality. The tidal front enters the gulf from the west and due to narrowing cross section and resonance, the tidal amplitude increases considerably, upstream of Vadinar.

Figure 15: Satellite picture of Vadinar coast in Gujarat, India

4.2 Water quality:

The yearly deviation of water temperature is between 20°C and 30°C, although the localized higher temperature goes upto 35°C which can result in isolated water pools formed in shallow intertidal depressions during low tide. Vertical profiles of temperature and salinity reveal a nearly homogeneous water column with no vertical stratification due to intense tidal-driven turbulence mixing.

4.3 Coral/coral reefs:

Stony corals of Gulf of Kachchh form the coral area in the northwest coast of Arabian Sea. The coral formations of the Gulf of Kutch are found between 22°20'N 22°40'N 69°70'E along the coast of Jamnagar district. 34 islands out of 42 in the Marine National Park bordering the southern shore of the Gulf support coral and coral reefs. Coral growth is scarce at present and perhaps not termed more as fats than fringing in the Salaya and Okha region. Growth at present is confined to intertidal sandstones or wave-cut eroded or shallow banks etc. Based on the classification of reefs, the reefs of the Gulf of Kutch are classified as fringing, platform reefs and patch reefs and coral pinnacles. The most northerly reefs or coral patches are found at

Munde Reef and Pirotan Island, but solitary corals are found as far as Dwarka on the Saurashtra coast.

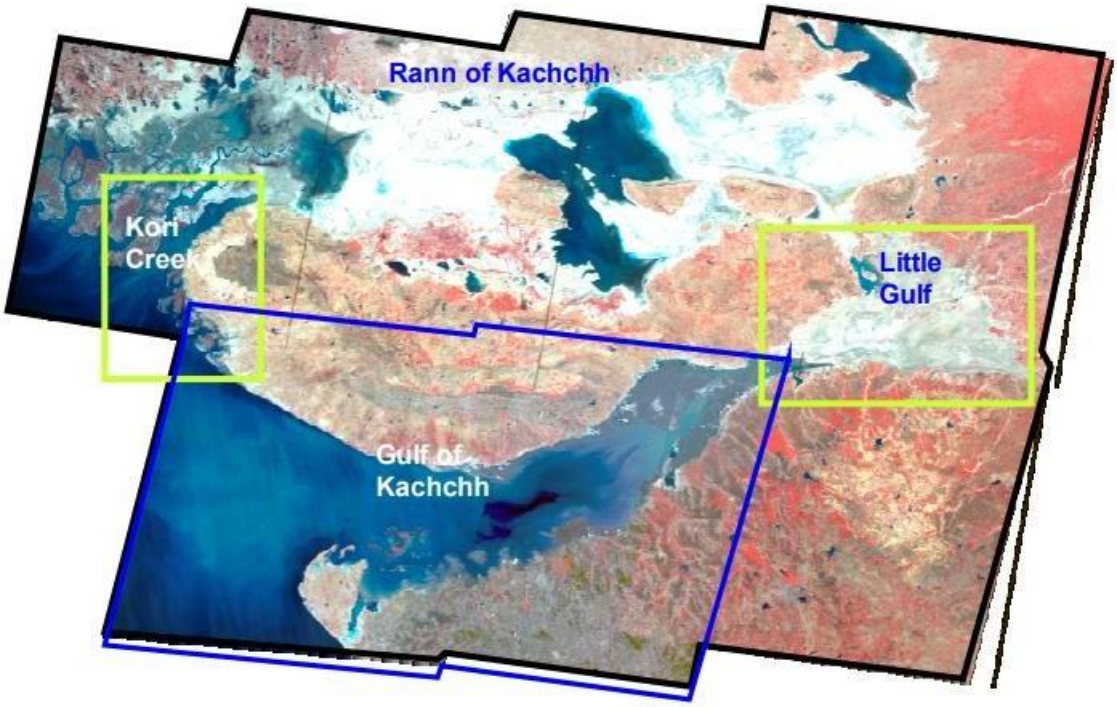


Figure 16: Satellite picture of Gulf of Kachchh, Gujarat

Coral species such as Turbinaria, Montipora, Fevia, Leptoria, Symphilia, Poritis, Leptastria, Hydropora, Goniopora, Goniastria etc. are found predominantly in the Gulf. Huge quantities of the dead horns of Acropora species are also found in the area that suggests that there could be a luxuriant growth of branched corals in the past.

The Gulf of Kutch flourishes in marine wealth and is considered as one of the biologically richest marine habitat along the west coast of India. The marine flora is highly varied and includes sand dune vegetation, mangroves, sea grasses, macrophytes and phytoplankton. The intertidal zone of the Gulf of Kutch is rich in biota and during high tide the coral thrive on less sunlight, corresponding to during the low tide, the corals are predominantly exposed to sunlight which causes stress to the coral-associated algae zooxanthellae and sometimes leave the coral, eventually causing death to coral due to non-requirement of food through photosynthesis.

CHAPTER 5: THE RESEARCH PROBLEM

Herein, the research gap found by considering various literature that is based upon coral and it is association in the intertidal reef habitat is addressed. The main aim of the report is to learn and identify the unique characteristics that makes the coral of Gulf of Kachchh thrive under severe stress condition.

5.1 Objectives:

1. To isolate the Symbiodinium cells from the corals in the intertidal reef flats of Gulf of Kachchh and optimize their culture conditions and growth.
2. To study the molecular diversity of Symbiodinium cells associated with the corals in the intertidal reefs, flats of Gulf of Kachchh.
3. To study the whole genome of the Symbiodinium cells associated with the corals to understand their evolutionary adaptations conducted to the intertidal reef conditions.

CHAPTER 6: REVIEW OF LITERATURE

1. The seasonal investigation of Symbiodiniaceae in broadcast spawning, *Acropora humilis* and brooding, by Suppakarn Jadang, Voranop Viyakarn, Yuki Yoshioka, Chuya Shinzato, Suchana Chavanich, PeerJ, The International Association for Biological Oceanography, 10: e13114 **(June 14, 2022)**

The density and diversity of Symbiodiniaceae associated with corals can be influenced by seasonal changes. This study provided the first annual investigation of Symbiodiniaceae density and diversity associated with *Acropora humilis* and *Pocillopora* cf. *damicornis* corals in the Gulf of Thailand using both zooxanthellae cell count and next-generation sequencing (ITS-1, ITS-2 regions) techniques, respectively. The results from this study indicated that zooxanthellae cell densities in both coral species differ significantly. The number of zooxanthellae was negatively correlated with the physical environment variable (light intensity). The diversity within *A. humilis* consisted of two genera, *Cladocopium* and a small amount of *Durusdinium* whereas *P.* cf. *damicornis* was found to be 100% associated with *Durusdinium* suggesting that each coral species may select their appropriate genus/species of Symbiodiniaceae in response to local environmental stressors. The results of this study provided some information on the coral-Symbiodiniaceae relationship between seasons, which may be applied to predict the potential adaptation of corals in localized reef environments.

2. Symbiodinium genomes reveal adaptive evolution of functions related to coral-dinoflagellate symbiosis by Huanle Liu1, Timothy G. Stephens1, Raúl A. González-Pech, Victor H.

Beltran, Bruno Lapeyre, Pim Bongaerts, Ira Cooke, Manuel Aranda, David G. Bourne, Sylvain Forêt, David J. Miller, Madeleine J.H. van Oppen, Christian R. Voolstra, Mark A. Ragan1 & Cheong Xin Cha, Communications biology article, 1:95 **(2018)**

Symbiosis between dinoflagellates of the genus Symbiodinium and reef-building corals forms the trophic foundation of the world's coral reef ecosystems. Here we present the first draft genome of Symbiodinium goreaui (Clade C, type C1: 1.03 Gbp), one of the most ubiquitous endosymbionts associated with corals, and an improved draft genome of Symbiodinium kawagutii (Clade F, strain CS-156: 1.05 Gbp) to further elucidate genomic signatures of this symbiosis. Comparative analysis of four available Symbiodinium genomes against other dinoflagellate genomes led to the identification of 2460 nuclear gene families (containing 5% of Symbiodinium genes) that show evidence of positive selection, including genes involved in photosynthesis, transmembrane ion transport, synthesis and modification of amino acids and glycoproteins, and stress response. Further, we identify extensive sets of genes for meiosis and response to light stress. These draft genomes provide a foundational resource for advancing our understanding of Symbiodinium biology and the coral-algal symbiosis.

3. Coral bleaching observations in the Gulf of Kachchh, India – A climate induced stress on the Scleractinians by Devanshi Joshi, Sandeep Munjpara, Upasana Banerji, Dishant Parasharayal, Gujarat Ecological Education and Research Foundation, 2:106-113 **(December 12, 2013)**

Climate driven changes have been identified in various ecosystems on earth and they result in either species adaptation or eradication. In order to get insight into such changes, it is necessary to assess their magnitude and rate which will lead to know the gravity of the threats on an ecosystem if any. The Gulf of Kachchh was considered as an area of low to moderate coral bleaching region hence this study was carried out to bring forth the coral health status in terms of coral bleaching. The event of large scale change-coral bleaching was observed in the Gulf of Kachchh during May and June, 2010. The incident was evinced by whitening of the coral colonies on the reefs of Narara and Poshitra in the Gulf of Kachchh. The present observations describe the incidents of mass coral bleaching, which affected a total of 19 scleractinian corals in addition to the other zooxanthellate cnidarians. Globally, a number of factors have been identified to induce the phenomenon but for this instance, elevated sea surface temperature is considered to be the responsible factor. The status of coral bleaching at different sites in the Gulf of Kachchh has been discussed.

CHAPTER 7: METHODOLOGY

7.1 Field trip:

For sample collection, a field visit was prior planned in the Jamnagar district of Gujarat, followed by sample collection from the Vadinar coast.

7.2 Site description:

The Gulf of Kachchh (22°15' to 23°40'N and 68°20' to 70°40'E is an east-west oriented indentation between Saurashtra and Kutch peninsulas. The Gulf of Kachchh is bordered by Kachchh district in the north and Jamnagar district in the south with Rajkot district covering a little portion of the gulf in its eastern side. The Gulf of Kachchh is 170 km long from Okha to inner gulf and is 75 km wide at its mouth with a water spread area of around 7300 km square and a volume of 2,20,000 m3. It narrows down abruptly with a distinct constriction at 70°20'E and Satsaida Beyt, and divides into a creek system often called the little gulf of Kutch. The gulf is characterized by numerous hydrographic irregularities like pinnacles as much as 10 m high. It is believed that the region of the Gulf of Kutch was a desert in the Pleistocene period with subsequently changed due to geological subsistence that allows the Arabian Sea to engulf the land. Its conical structure leads to elevated tidal levels, especially in the inner gulf. The high tidal flux covers vast low-lying areas of about 1,500 km2, comprising a network of creeks, estuaries and mudflats in the interior regions. The creek system consists of three main creeks, namely Kandla, Hansthal and Nakti and the little Gulf of Kachchh interconnected through many large and small creeks. The southern part of the gulf has diverse physical conditions and large marine ecosystem which supports the huge health of marine life. The gulf environment is ecologically very sensitive as it supports vast areas under mangroves, coral reefs, mudflats and their associated ecosystem.

7.3 Sample collection:

Colonies of the scleractinian coral, Siderastrea, Porites, and Favites were collected using snorkelling at depths of 2–3 m from inside the Gulf of Kachchh, Vadinar, a small coastal town located in the Devbhoomi Dwarka district of Gujarat. The samples were transported in seawater with aeration and covered to avoid overexposure to solar radiation and to maintain temperatures relatively constant. Within 1 h of collection the samples were brought to the laboratory. If the samples could not be processed immediately they were maintained in a flowing seawater aquarium and processed within 24 h of collection. Prior to the processing of the samples, the surfaces and undersurfaces of the colonies were rinsed with 0.45 um filtered seawater to remove contaminating organism.

Figure 17: A snorkeller

7.4 Isolation and culturing of Symbiodinium:

7.4.1. Separation of coral and symbionts:

Coral tissue lies as a thin veneer over a calcium carbonate skeleton with the algal cells residing within the endodermal cells of the invertebrate coral host. The Water Pik (a dental device that uses a re-circulating pump to produce a jet of water under pressure to dislodge particles from between teeth) has been routinely used to remove the coral tissue from its

skeleton, since its first description). This method generates a large volume (50 mL–2 L) of ‘‘blastate’’ and after centrifugation of the sample, the pellet, containing the symbionts, has generally been used to determine parameters associated with the algal cells, such as cell numbers, specific growth rate, mitotic index and chlorophyll concentrations or more recently, genetic classification. The composition of the supernatant and pellet has also been analyzed for carbon, nitrogen, total proteins, lipids and carbohydrates; however, the large volume of blastate produced is not conducive to DNA analysis of the host tissue. To produce a more concentrated slurry of coral tissue and symbiotic algae than that generated by the Water-Pik method, an artist's airbrush, modified to deliver a jet of high-pressure air and seawater onto the coral surface has been also used but the volumes are generally 5–10 mL. To reduce the volume even further, razor blade scrapes have also been used and this method was found to be efficient due to the low volume produced and time saved for the extraction of genomic DNA from coral and algal components simultaneously in comparison with the other methods. A known area of coral was delimited using a corer for three replicate samples and scraped with a single edged razor blade and the scrapings resuspended using a motor-driven pestle (Kontes) in an extraction buffer (EB) of 5 mM EDTA in sterilized 0.45 um filtered seawater, or 100 mM EDTA, pH 8.0, 10 mM Tris (pH 7.5) or 100 mM Tris, 20 mM EDTA. Three replicates were used in each buffer condition. The mixture was ground to homogeneity in a glass-glass tissue grinder and placed on ice. Algal cell numbers were determined in three subsamples with four replicate counts each by optical microscopy with a Bright Line counting chamber (hemocytometer) with an improved Neubauer ruling pattern (Hausser Scientific). Light microscopic examination also indicated no observable damage to the algal cells during this step. The homogenate was spun for 5 min at 16,000 g at 22 degrees Celsius to separate the algal cells from the animal tissue. The supernatant was decanted and placed on ice and the pellet was resuspended in EB. Subsamples of the supernatant were taken for the determination of algal cell numbers by four replicate hemocytometer counts. The pellet contained algal cells surrounded by host cell membranes, which were removed by resuspension in EB and SDS to a final concentration of 0.1%. The mixture was gently mixed, left on ice for 10 min with occasional mixing and centrifuged at 16 000 g for 10 min at 22 degrees Celsius. The supernatant was discarded and the pellet washed in EB and centrifuged, repeating this process for a total of three times, at which point microscopic observation indicated that the algal cells were devoid of host cell membranes. Subsamples of the pellet were taken for the determination of algal cell numbers by four replicate counts in a hemocytometer.

With the coral tissue slurry, filtration with mesh in a 50 ml or 15 ml centrifuge tubes were performed. Centrifugation of the pitcher was carried out at 5000 A for 5 minutes. After centrifugation, the supernatant was discarded and the pellets were re-suspended in 2 ml of F/2 media and whole contents were transferred to Guillard's (F/2) Marine Water

Enrichment Solution (1x, liquid, plant cell culture tested). Further, 2 ml of antibiotic cocktail containing Kanamycin (50 ug/ml), Ampicillin (100 ug/ml) and Streptomycin (50 ug/ml) were also added and the media was changed continuously every 10 days for a span of 2 month. Their growth was monitored under the microscope.

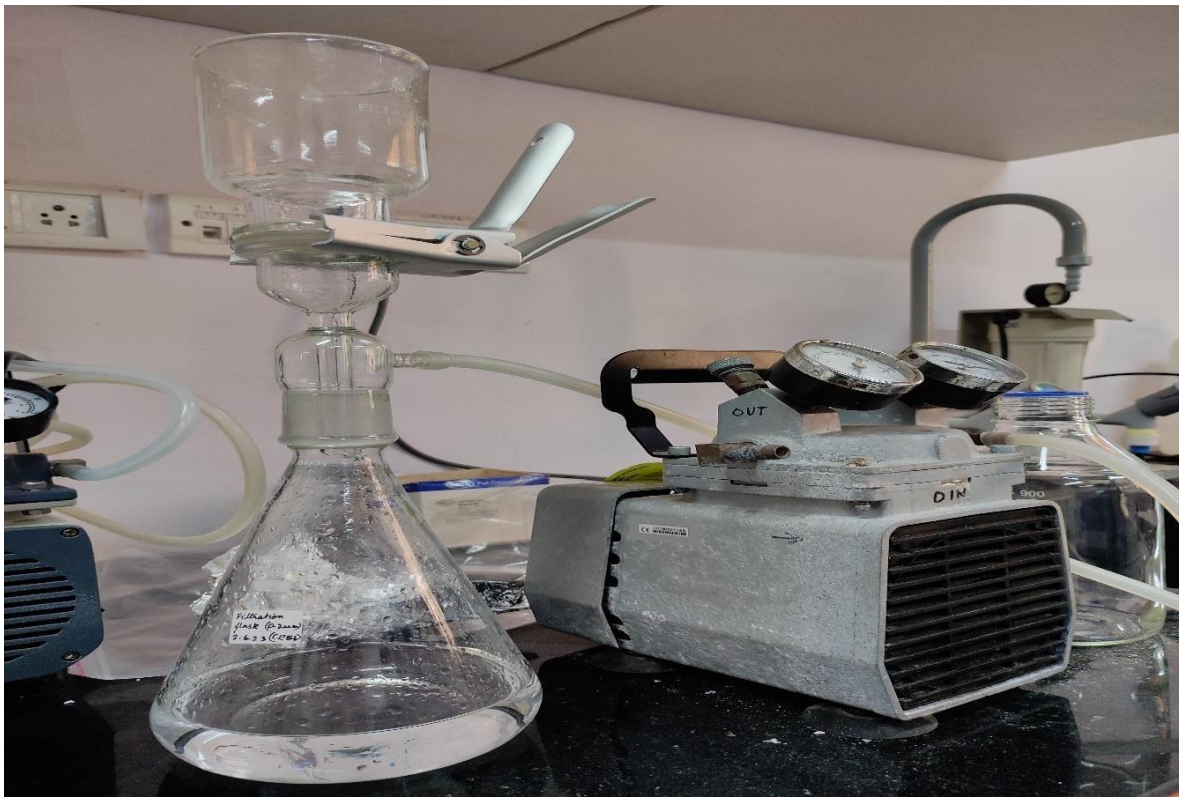


Figure 18: A filtration unit to filter the sea water

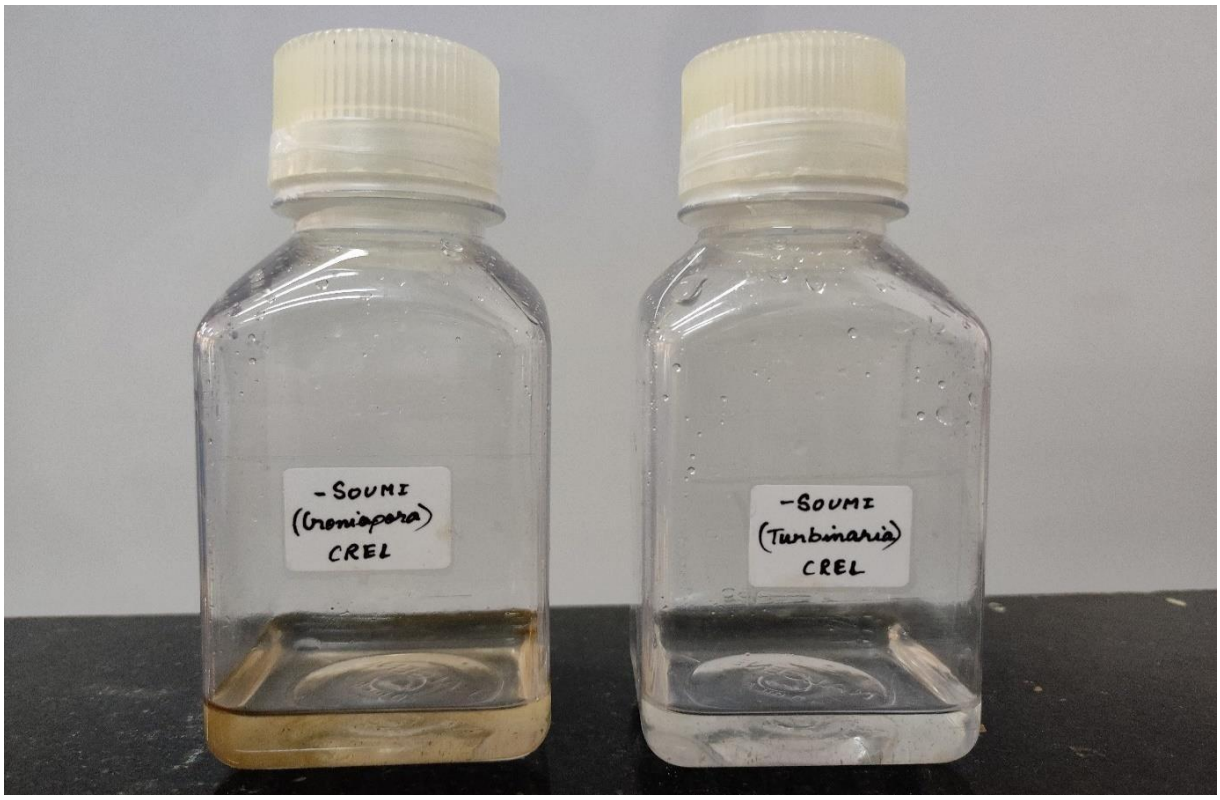


Figure 22: Nalgene bottle containing coral species and antibiotics to observe growth

Unique cells of Symbiodinium were selected under microscope for culturing and grown in 6-well plate from which unique cells were transferred into agar plates. Throughout the experiment, cells were cultured in F/2 media containing Kanamycin (50 ug/ml), Ampicillin (100 ug/ml) and Streptomycin (50 ug/ml) and growth was routinely monitored under microscope to ensure colony formation.

7.4.2 Determination of zooxanthellae density:

For zooxanthellae density determination, the decalcification method wherein preserved coral branches were placed in a glass container with 5% HCl solution until the coral skeleton was completely dissolved (~4–7 days depending on size and species) was incorporated. The remaining tissues were rinsed and preserved in 70% ethanol. Three pieces of fixed tissue were cut into 1 × 1 cm sections and then preserved with 70% ethanol. Prepared samples were homogenized using a Nissei ACE homogenizer for 5 min until the tissue was well-mixed. Subsamples (2.5 µl) of the coral tissue homogenate were immediately placed on each chamber of a Neubauer-improved bright line haemocytometer to count the number of zooxanthellae under 40x magnification. Four corner squares of each chamber were counted as one replication. To calculate the mean zooxanthellae density per cm^2, the average total of zooxanthellae cell counted on each square was multiplied by 10^4ml (the volume of each chamber) and the dilution then divided by the coral tissue area (cm^2). The mean zooxanthellae density per cm^2 is calculated by average total of zooxanthellae cell counted on each square multiplied by 10^4 ml

(volume of each chamber) multiplied by dilution, followed by division of the coral tissue area (cm^2).

Figure 23: Decalcification of coral

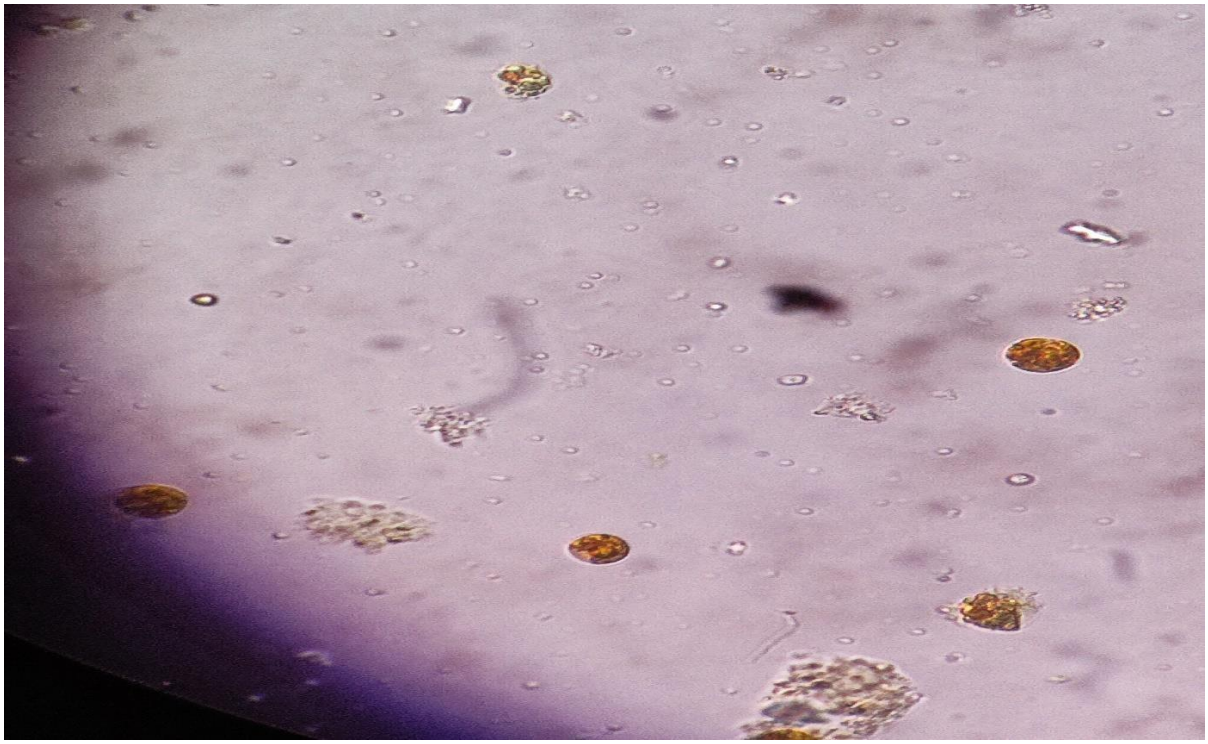


Figure 24: Zooxanthellae cells observed in 100x magnification under light microscope

7.5 Genomic DNA isolation:

Small pieces of coral preserved in 95% ethanol were dried and ground using a pestle and mortar to create a coarse powder. Each powdered coral was used to extract the genomic DNA of zooxanthellae using a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions with minor modification) and the procedure was performed in

duplicates. Crude DNA was analyzed using a NanoDrop 2000c (Thermo Fisher Scientific, Waltham, MA, USA) to examine the quality and concentration of DNA. Extracted DNA was used as a PCR template to amplify the DNA of the Symbiodiniaceae. The PCR amplifications were performed using primers for the internal transcribed spacers 1 and 2 (ITS-1 and ITS-2) genes. The targeted ITS-1 and ITS-2 regions were amplified using the specific primers for Symbiodiniaceae species identification including forward: r18Sf (5′-CGCTCTTCCGATCTCTGGAAAGTTTCATGAACCTTA T3-′) and reverse: Sym28Sr-1st (5′-TGCTCTTCCGATCTGACCTTGTRTGACTTCATGCTA -3′). PCR was carried out using Thermo Fisher Veriti 96-Well Thermal Cycler™ with a reaction volume of 25 µl mixture for each sample which consisted of readily available PCR mastermix of 12.5 µl, 0.5 µl of each forward and reverse primer, 8.5 µl of nuclease-free, RNase-free, DNase -free water (Sigma® Life Science) and 3 µl of DNA template. The PCR conditions were set at 15 min at 94 °C, followed by 35 cycles of 94 °C for 30 s, 57 °C for 90 s, 72 °C for 90 s, and a final extension step of 10 min at 72 °C. After PCR, each amplicon was visualized under 1.5% agarose gel electrophoresis along with 1000 bp ladder using SYBR-Green as dye to check the target size of the DNA. Also, 22 µl of each amplicon along with 4 µl of bromophenol blue as DNA gel loading dye was loaded into each well for further process. Besides, 2 µl of the amplicon was quantified using Nanodrop Spectrophotometer and readings were noted. Finally, the PCR products were cleaned and purified by directly excising the gel using a sharp scalpel and following using the QIAquick Gel Extraction Kit by following the manufacturer's protocol. Each purified sample was then used for the second PCR. Second PCR products were run in a 1.5% agarose gel to check the targeted size of DNA and thermal mixture condition was run same as the previous run.

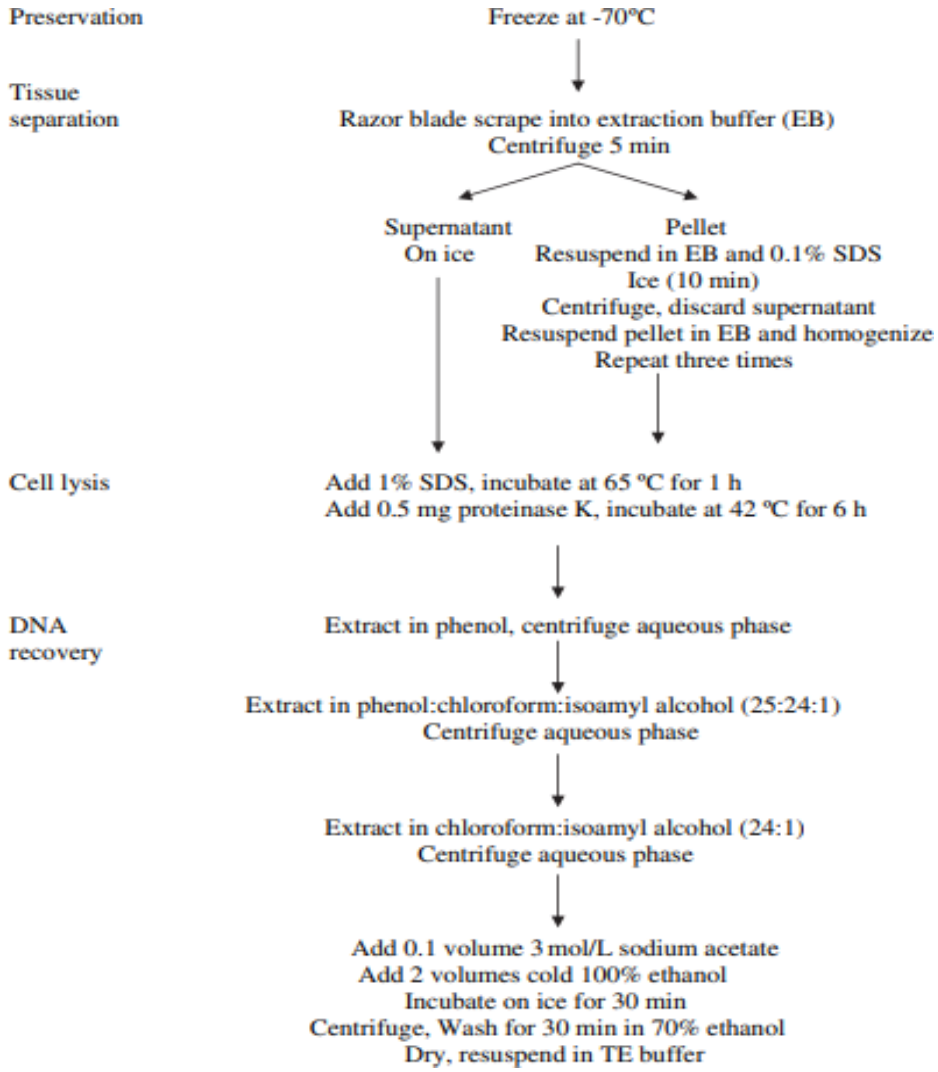


Figure 25: Flow chart of the optimized protocol for the extraction of DNA from the coral host and of the symbiotic algae. All centrifugation steps are run for 10 minutes at 16,000 g unless otherwise noted

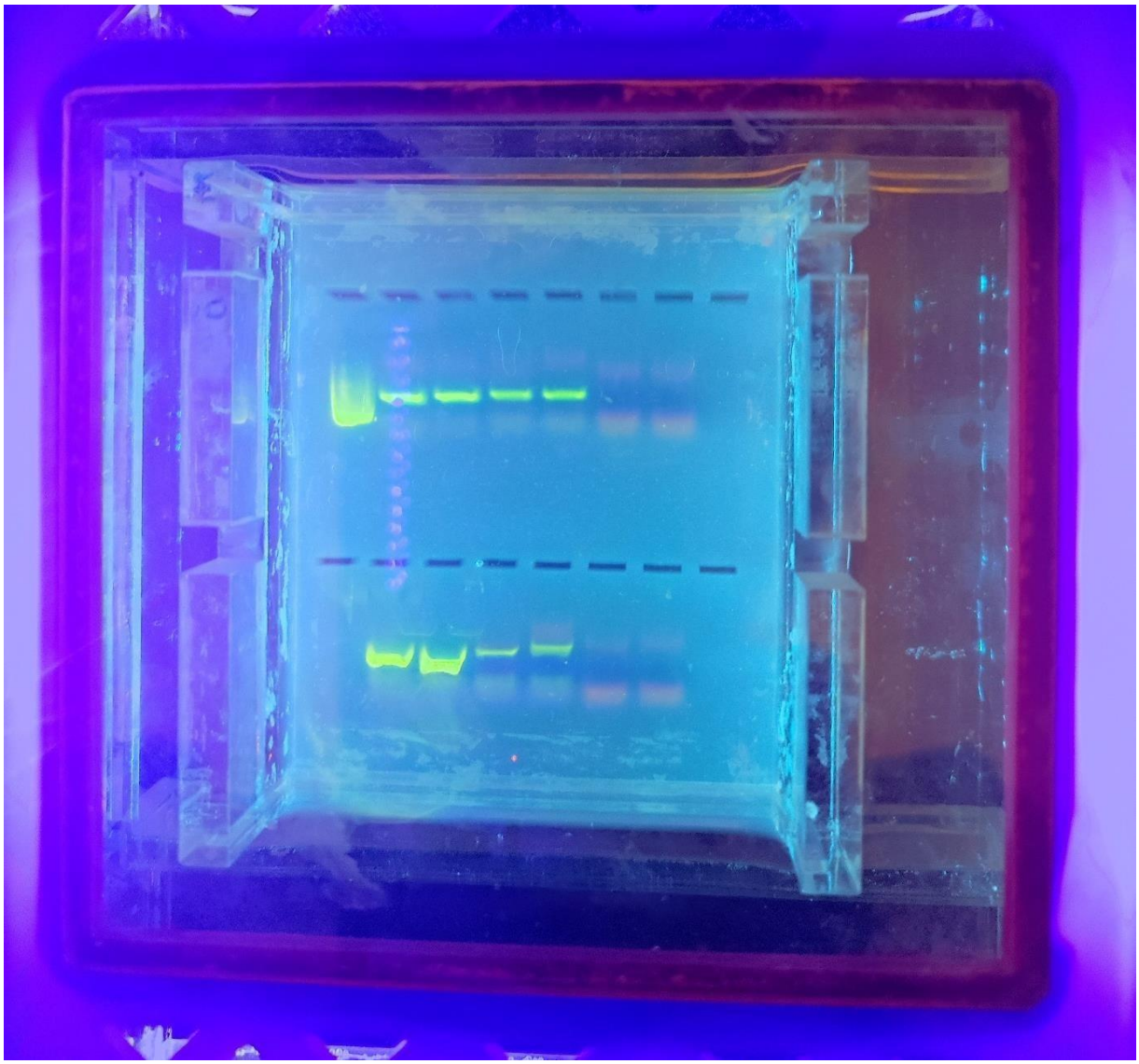


Figure 26: Electrophoresis gel unit having DNA with SYBR green dye after successful run

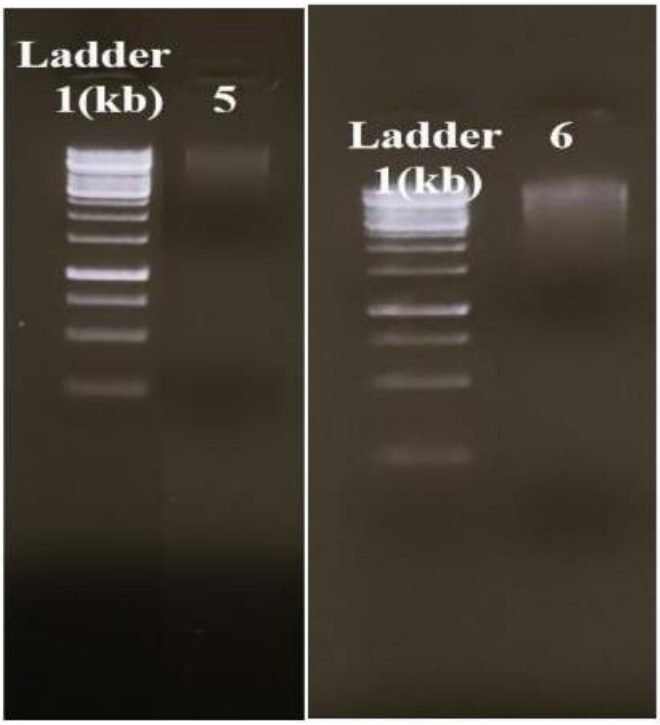


Figure 27: Genomic DNA run

7.6 Molecular identification of Symbiodinium:

The polymerase chain reaction (PCR) was originally developed in 1983 by the American biochemist Kary Mullis. He was awarded the Nobel Prize in Chemistry in 1993 for his pioneering work. It is used in molecular biology to make many copies of amplified small sections of DNA or a gene. Using PCR it is possible to generate thousands to millions of copies of a particular section of DNA from a very small amount of DNA. PCR is a common tool used in medical and biological research labs. It is used in early stages of processing DNA for sequencing, for detecting the presence or absence of a gene to help identify pathogens during infection and when generating forensic DNA profiles from tiny samples of DNA.

PCR is a biochemical process capable of amplifying a single DNA molecule into millions of copies in a short time. Amplification is achieved by a series of three steps:

(1) denaturation, in which double-stranded DNA templates are heated to separate the strands;

(2) annealing, in which short DNA molecules called primers bind to flanking regions of the target DNA; and

(3) extension, in which DNA polymerase extends the 3′ end of each primer along the template strands. These steps are repeated (“cycled”) 25–35 times to exponentially produce exact copies of the target DNA.

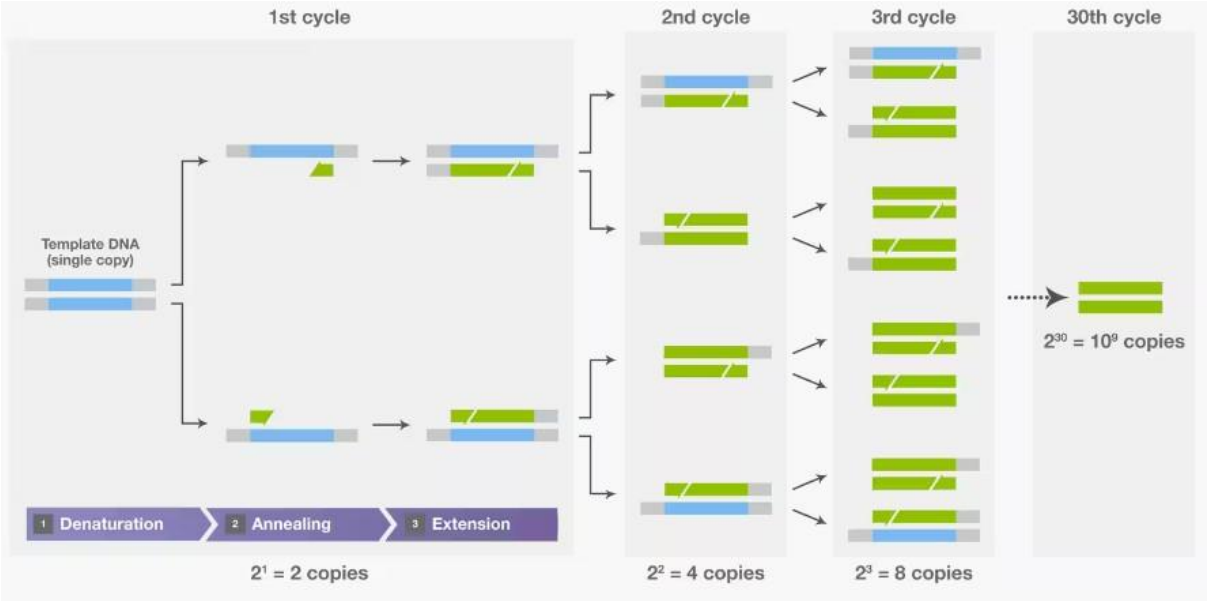


Figure 28: Three steps of PCR – denaturation, annealing, and extension

PCR is a three-step process that is carried out in repeated cycles. The initial step is the denaturation, or separation, of the two strands of the DNA molecule. This is accomplished by heating the starting material to temperatures of about 95 °C (203 °F). Each strand is a template on which a new strand is built. In the second step the temperature is reduced to about 55 °C (131 °F) so that the primers can anneal to the template. In the third step the temperature is raised to about 72 °C (162 °F), and the DNA polymerase begins adding nucleotides onto the ends of the annealed primers. At the end of the cycle, which lasts about five minutes, the temperature is raised and the process begins again. The number of copies doubles after each cycle. Usually 25 to 30 cycles produce a sufficient amount of DNA.

In the original PCR procedure, one problem was that the DNA polymerase had to be replenished after every cycle because it is not stable at the high temperatures needed for denaturation. This problem was solved in 1987 with the discovery of a heat-stable DNA polymerase called *Taq*, an enzyme isolated from the thermophilic bacterium *Thermus aquaticus*, which inhabits hot springs. *Taq* polymerase also led to the invention of the PCR machine.

DNA polymerases are critical components in PCR, since they synthesize the new complementary strands from the single-stranded DNA templates. All DNA polymerases possess 5′→ 3′ polymerase activity, which is the incorporation of nucleotides to extend primers at their 3′ ends in the 5’ to 3’ direction.

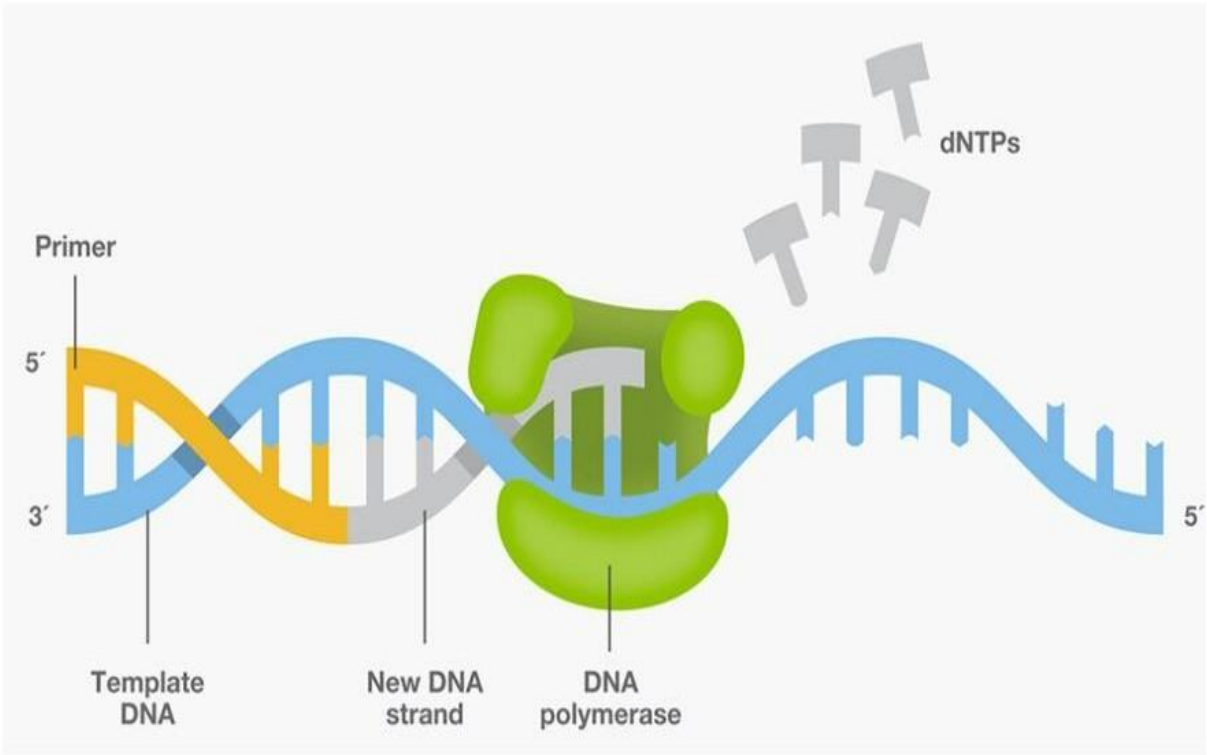


Figure 29: DNA polymerase extending the 3` end of a PCR primer in the 5` to 3` direction

In the early days of PCR, the Klenow fragment of DNA polymerase I from *E. coli* was used to generate the new daughter strands. However, this *E. coli* enzyme is heat-sensitive and easily destroyed at the high denaturing temperatures that precede the annealing and extension steps. Thus, the enzyme needed to be replenished at the annealing step of each cycle throughout the process.

The discovery of thermostable DNA polymerases proved to be an important advancement, opening tremendous opportunities for the improvement of PCR methods by enabling longer-term stability of the reactions. One of the best-known thermostable DNA polymerases is *Taq* DNA polymerase, isolated from the thermophilic bacterial species *Thermus aquaticus* in 1976 [5,6]. In the first report in 1988 [7], researchers demonstrated *Taq* DNA polymerase's retention of activity above 75°C, making continuous cycling without manual addition of fresh enzyme possible, and thus enabling workflow automation. Furthermore, compared to *E. coli* DNA polymerase, *Taq* DNA polymerase produced longer PCR amplicons with higher sensitivity, specificity, and yield.

Taq DNA polymerase is an enzyme taken from the heat-loving bacteria?*Thermus aquaticus*. This bacteria normally lives in hot springs so can tolerate temperatures above 80ºC. The bacteria's DNA polymerase is very stable at high temperatures, which means it can withstand the temperatures needed to break the strands of DNA apart in the denaturing stage of PCR. DNA polymerase from most other organisms would not be able to withstand these high temperatures, for example, human polymerase works ideally at 37˚C (body temperature). 72˚C is the optimum temperature for the Taq polymerase to build complementary strand.

Although *Taq* DNA polymerase significantly improved PCR protocols, the enzyme still presented some drawbacks. *Taq* DNA polymerase is relatively unstable above 90°C during denaturation of DNA strands. This is especially problematic for DNA templates with high GC content and/or strong secondary structures that require higher temperatures for separation. The enzyme also lacks proofreading activity; therefore, *Taq* DNA polymerase can mis-incorporate nucleotides during amplification. Where sequence accuracy is critical, PCR amplicons with errors are not desirable for cloning and sequencing. In addition, the error-prone nature of *Taq* DNA polymerase contributes to its inability to amplify fragments longer than 5 kb in general. To overcome such shortcomings, better-performing DNA polymerases are continually being developed to harness the power of PCR across a variety of biological applications

Multiplex PCR employs different primer pairs in the same reaction for simultaneo us amplification of multiple targets. This type of PCR often requires extensive optimization of annealing conditions for maximum amplification efficiency of the different primer–template systems and is often compromised by nonspecific PCR artifacts. A stringent hot-start procedure and specially optimized buffer systems are absolutely crucial for successful multiplex PCR.

Compared with standard PCR systems using only 2 primers, an additional challenge of multiplex PCR is the varying hybridization kinetics of different primer pairs. Primers that bind with high efficiency could utilize more of the PCR reaction components, thereby reducing the yield of other PCR products. This often results in unamplified DNA sequences and absence of expected PCR products.

A thermal cycler is an instrument that automates temperature cycling and incubation times for PCR. PCR was a laborious process involving the transfer of samples between water baths of different temperatures, and requiring precise timing of each step. The thermal cycler, together with the discovery of *Taq* DNA polymerase, made automation of PCR a reality. The first automated thermal cycler for PCR was introduced to the market by PerkinElmer and Cetus as a joint venture in 1985. Since then, improvements have been made in the utility, design, temperature control, and cycling speed of thermal cyclers Thermal cyclers also paved the way for the development of quantitative PCR instruments that combine PCR amplification with real-time detection of PCR product accumulation (learn more about quantitative PCR.

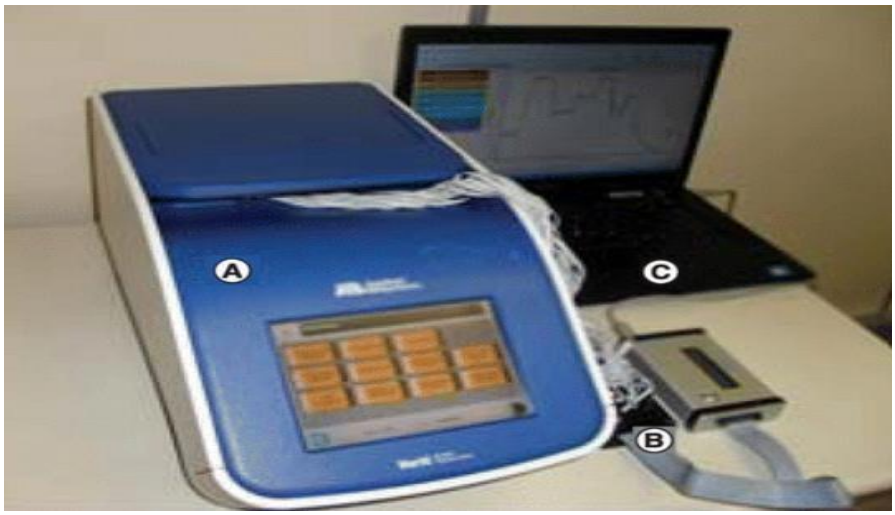


Figure 30: A thermal cycler

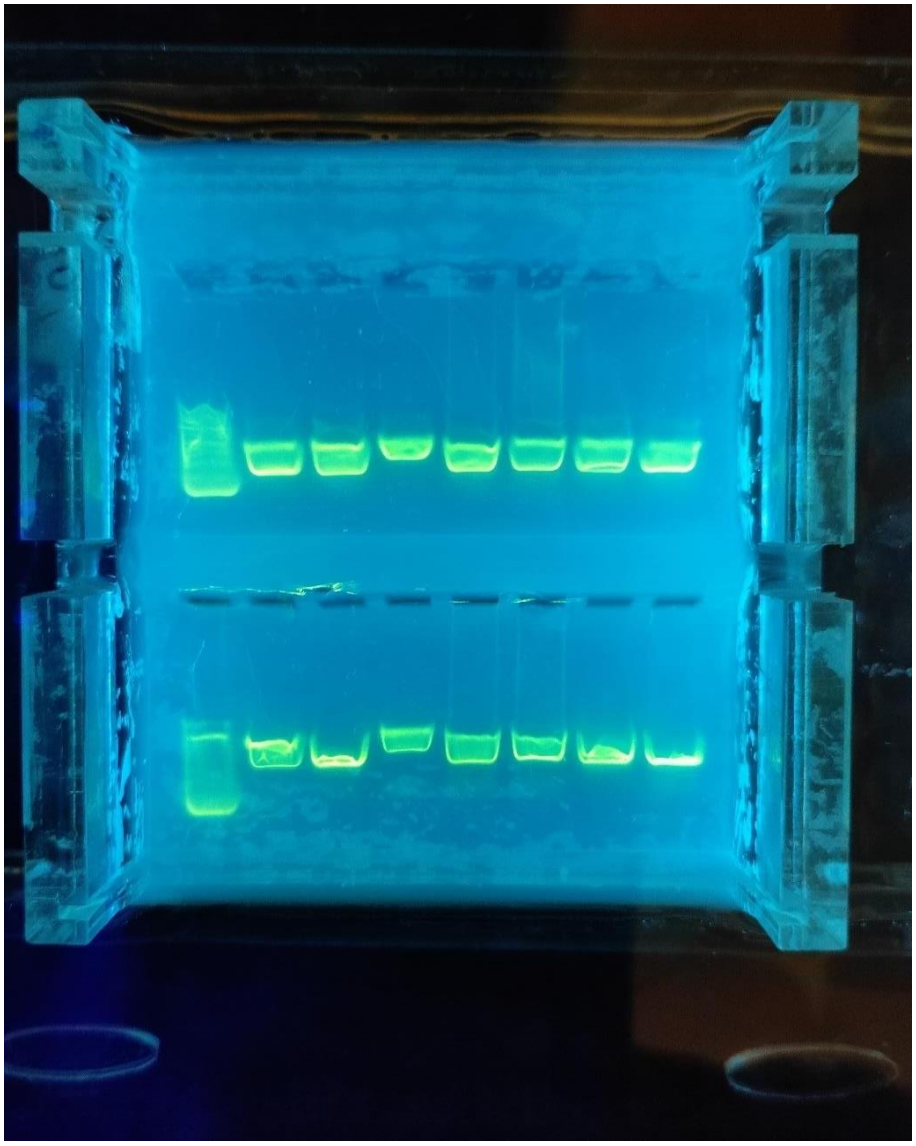


Figure 31: Electrophoresis gel loaded with amplicons

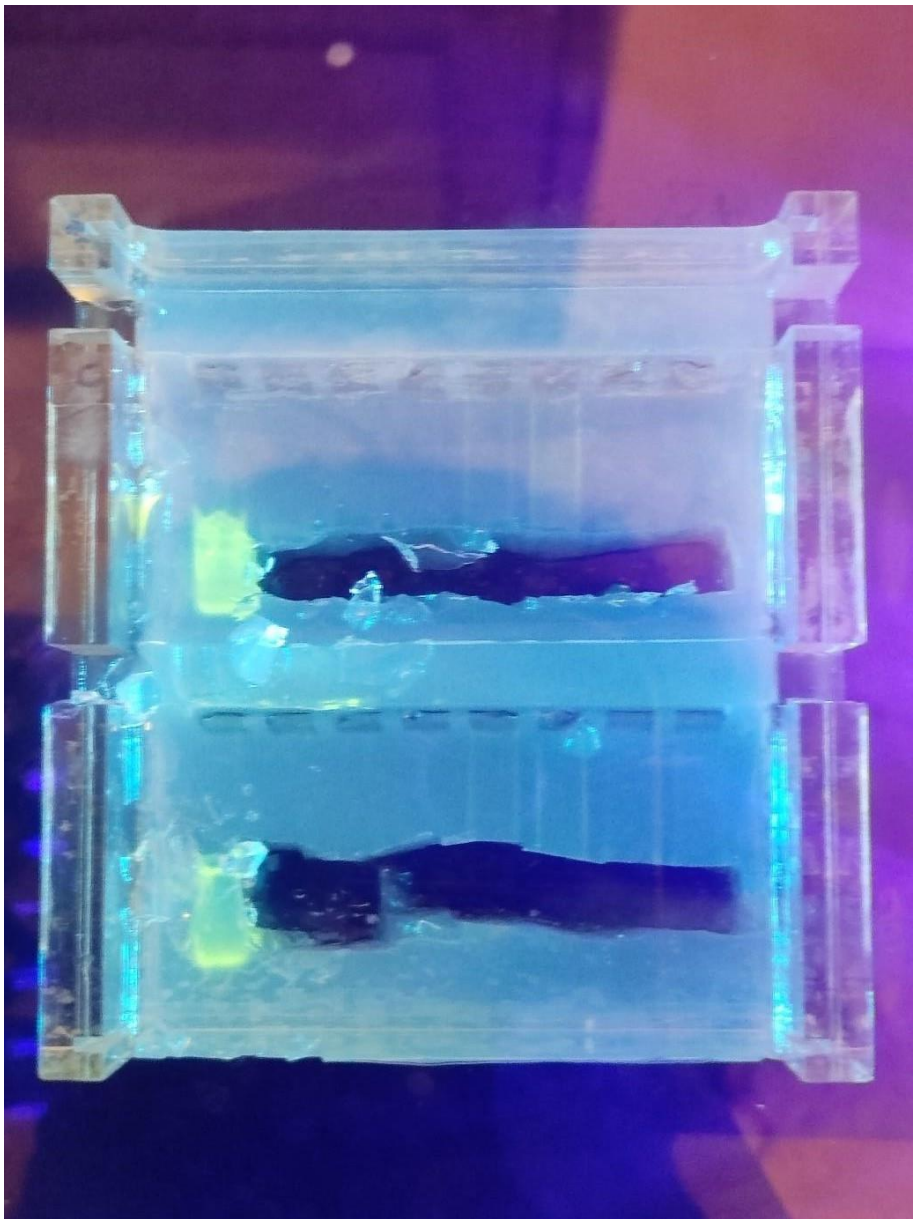


Figure 32: Electrophoresis gel after excision of amplicons for PCR cleanup/purification steps

7.7 Whole genome sequencing:

Whole-genome sequencing (WGS) is a comprehensive method for analyzing entire genomes. WGS refers to the construction of the complete nucleotide sequence of a genome (~3.2 billion base pairs in humans), and provides a powerful tool to obtain greater insight into the genetic variability. Whole genome sequencing reveals the complete DNA make-up of an organism, enabling us to better understand variations both within and between species. This in turn allows us to differentiate between organisms with a precision that other technologies do not allow. In comparison to targeted sequencing, WGS explores a much larger sequence space to generate a huge genetic diversity. Though the cost-effectiveness of this technique is an issue, the WGS

technique provides a high taxonomic resolution and shows a complete microbial profile of the community besides generating information on the functional genes present in the microbes. As huge data is generated after the WGS approach, it is of huge importance to select an appropriate computational technique to analyze the sequenced data. The feasibility of WGS analysis is under the support of next generation sequencing (NGS) technologies, which require substantial computational and biomedical resources to acquire and analyze large and complex sequence data.

Each successful PCR amplicon was pooled together in an equimolar concentration for sequencing. Pooled samples were cleaned, followed by DNA libraries then were quantified to examine the concentration and average DNA fragment size (bp). Paired end DNA libraries was used and sequencing on an Illumina Mi-Seq platform by the Redcliffe Labs, Bengaluru, India.

The sequencing data was visualized using MEGA11 software for each of the individual coral species. After that, MUSCLE software was used to obtain ClustalW alignment and establish a phylogenetic of the clades based on the sequencing data available.

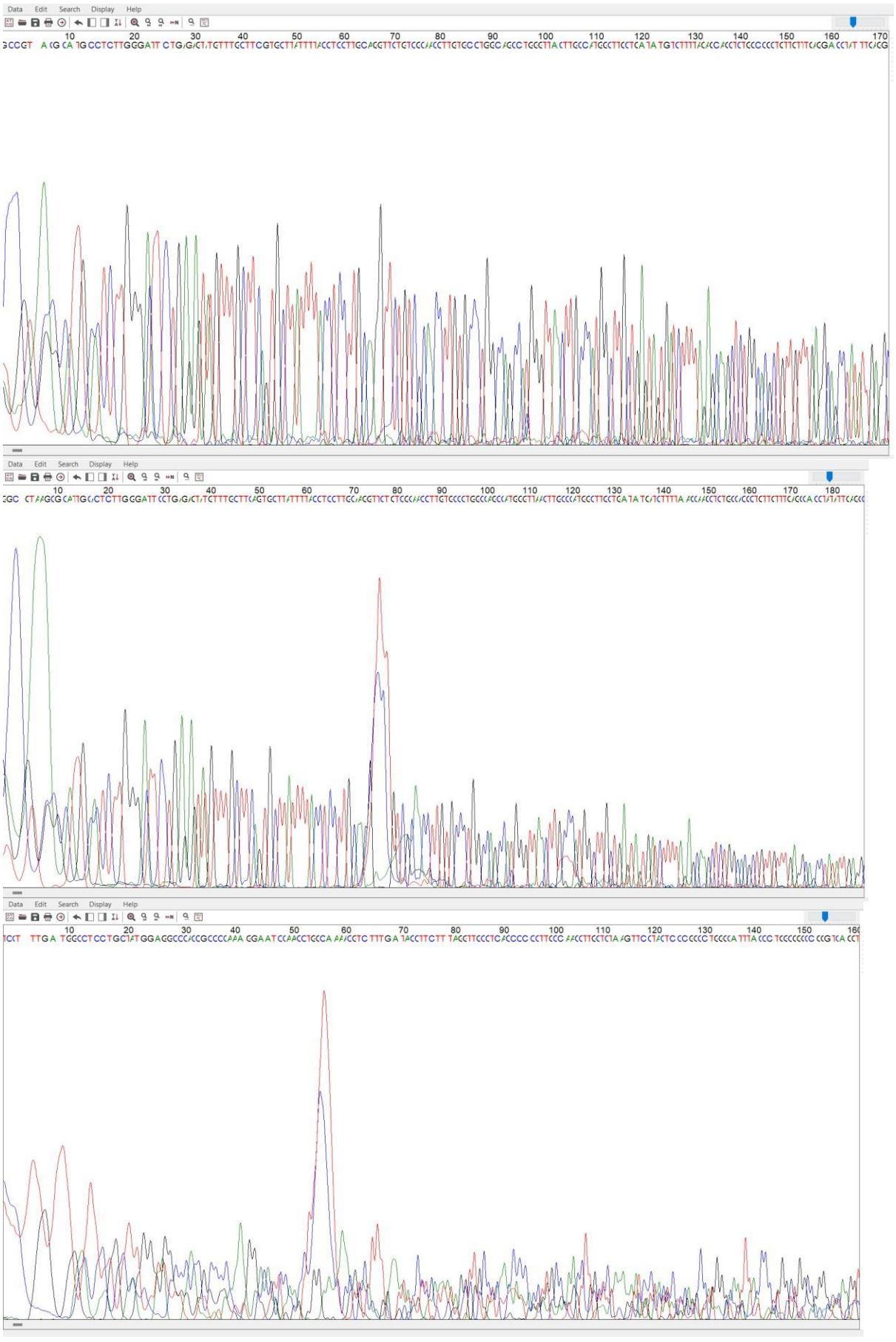
Sanger sequencing is a method of DNA sequencing that involves electrophoresis and is based on the random incorporation of chain-terminating dideoxin impurities by DNA polymerase during in vitro DNA replication. After first being developed by Frederick Sanger and colleagues in 1977, it became the most widely used sequencing method for approximately 40 years. It was first commercialized by Applied Biosystems in 1986. More recently, higher-volume Sanger sequencing has been replaced by next-generation sequencing methods, especially for large-scale automated genome analysis. However, the Sanger method remains in wide use for smaller-scale projects and for validation of desequencing results. It still has the advantage over short-rate sequencing technologies like Illumina in that it can produce DNA sequencing rates of greater than 500 nucleotides and maintains a very low error rate with accuracy around 99.99%.
In Sanger sequencing, a template DNA strand is first denatured into single strands. These single strands then serve as templates for the synthesis of a complementary DNA strand using DNA polymerase and a primer. The primer is typically labeled with a fluorescent dye.

In addition to the four deoxynucleotide triphosphates (dNTPs), the reaction mixture also contains low concentrations of dideoxynucleotide triphosphates (ddNTPs), which lack the 3'-OH group necessary for the addition of another nucleotide. The ddNTPs are labeled with different fluorescent dyes.

During DNA synthesis, both dNTPs and ddNTPs are incorporated into the growing DNA strand. However, when a ddNTP is added, no further synthesis can occur due to the lack of the 3'-OH group. As a result, the DNA fragments terminate at the specific nucleotide where the ddNTP was incorporated.

The reaction mixture is then subjected to gel electrophoresis, which separates the synthesized DNA fragments by size. The fragments are detected and visualized by laser-induced fluorescence. The Fluorescent signals from the labeled ddNTPs are detected, and the order of the nucleotides in the DNA strand is determined by analyzing the different-colored signals.

Sanger sequencing can sequence DNA fragments up to several hundred nucleotides in length and has been critical for many genomic sequencing projects and research studies. However, it is a labor-intensive and time-consuming technique, and newer methods like next-generation sequencing have largely replaced it for large-scale DNA sequencing.
The result achieved from Sanger sequencing is analysed using BLAST software, and visualized using MEGA11 database, and tree data for the phylogenetic tree were generated using MUSCLE software with ClustalW alignment.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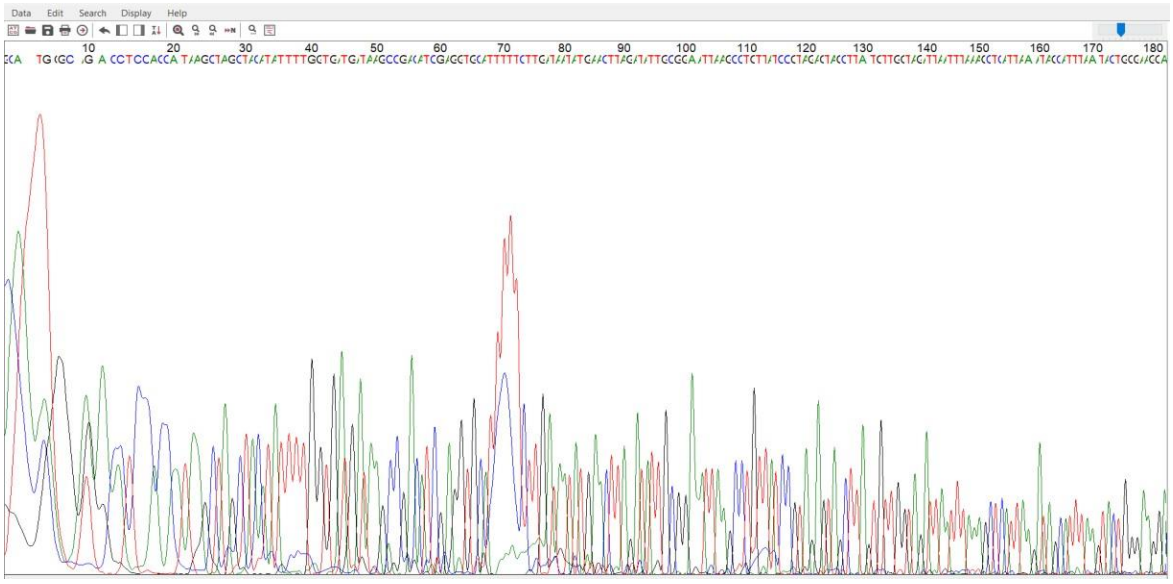

Figure 33: Sequencing result for Siderastrea, Favites, Porites, species with ITS primer bi-directional

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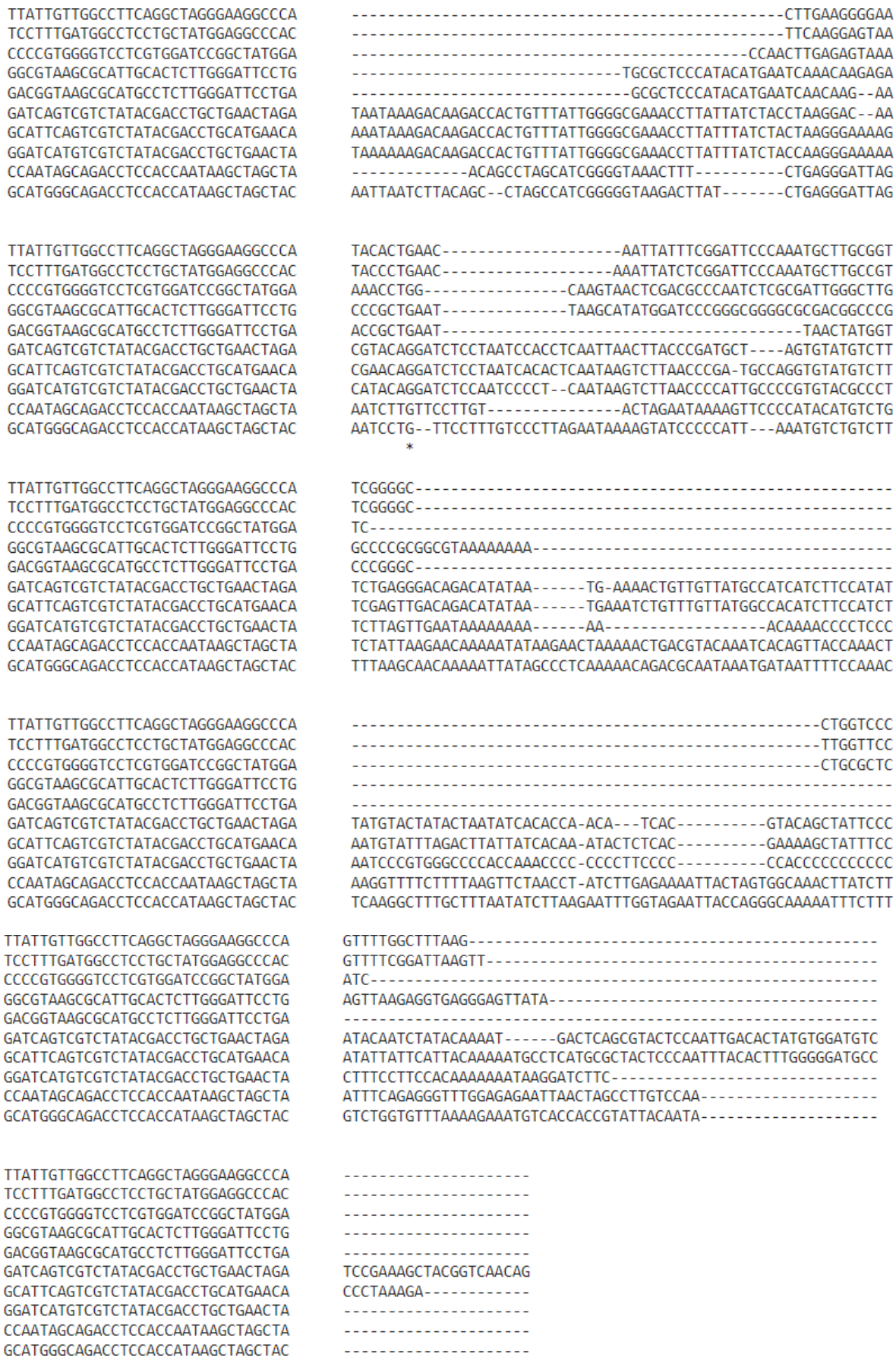
CLUSTAL multiple sequence alignment by MUSCLE (3.8)


TTATTGTTGGCCTTCAGGCTAGGGAAGGCCCA      ------------TAGGTTGCCCCACCCCCCTTG---CCAACCTGGCCCAAGTTC-CTA--
TCCTTTGATGGCCTCCTGCTATGGAGGCCCAC      --------------GGTTGCCTCACCCCCCTTG---CCAACCTTGCTCTAAGTTCCTA--
CCCCGTGGGGTCCTCGTGGATCCGGCTATGGA      --------------GGTTGC----TCACCCCAAGGGCCAACCTTGTCAAA----------
GGCGTAAGCGCATTGCACTCTTGGGATTCCTG      -------CGCAACCTTGTGCCCTGGCCAGCCATGGGTTAACTTGCCCATGGCTTGCTGAT
GACGGTAAGCGCATGCCTCTTGGGATTCCTGA      -----------ACCTTGTGCCCTGGCCAGCCATGGGTTAACTTGCCCATGGCTTGCTGAG
GATCAGTCGTCTATACGACCTGCTGAACTAGA      ---------AGATATGCTGTCTAATATACT------CTAACTAAAGGAAA----------
GCATTCAGTCGTCTATACGACCTGCATGAACA      TCAGTACAAAGATATGCTGTCTAAGTAATACAT---CTAACTAAAGGACA----------
GGATCATGTCGTCTATACGACCTGCTGAACTA      ---GTACAAAGATATGCTGTCTAAGTATACT-----CTAACTAAAGGACA----------
CCAATAGCAGACCTCCACCAATAAGCTAGCTA      -ATATGAACTTAGATATTGCGGAATTAAGCCTG---TTATCCGTAGAGTAGCTTAGTGTT
GCATGGGCAGACCTCCACCATAAGCTAGCTAC      --TATGAACTTAGATATTGCGGAATTAAGCCTG---TTATCCGTAGAGTAGCTT-ATGTT
                                                    **                  * *

TTATTGTTGGCCTTCAGGCTAGGGAAGGCCCA      ---------CTCCCACCCT----GGGCATTTACCCTGGCGGGCCGGGTAAGGTTCCGCAA
TCCTTTGATGGCCTCCTGCTATGGAGGCCCAC      ---------CTCCCGGCCT----GGGCATTTACCCTGGCGGGCCGGGTCAGGTTGCGCCA
CCCCGTGGGGTCCTCGTGGATCCGGCTATGGA      ----------TCCTAACTTATCCTGGAAATAACCCTGGT--GCCGGATTAGTTGG-----
GGCGTAAGCGCATTGCACTCTTGGGATTCCTG      ATGAT----CTTTTAAGC-----------AAGCTCTGGC--ACGCTGTTGTTTGAGGCAC
GACGGTAAGCGCATGCCTCTTGGGATTCCTGA      TAGTGATC-TTTTAGAGC-----------AAGCTCTGGC--ACGCTGTTGTTTGAGGCAC
GATCAGTCGTCTATACGACCTGCTGAACTAGA      -AAAGACC-CTTTGAAGCTTTTAGGGTAAAA--TTTGAT--GCTTTTTTAGCTTAGGCCG
GCATTCAGTCGTCTATACGACCTGCATGAACA      AAAAGACC-CTTTGAAGCTTTATAGGGAAAAACTTTGAT--GCTTTTTTAGCTTAGGCCG
GGATCATGTCGTCTATACGACCTGCTGAACTA      AAAAGACC-CTTTGAAGCTTTATAGGGAAAAACTTTGAT--GCTTTTTTAGCTTAGGCCG
CCAATAGCAGACCTCCACCAATAAGCTAGCTA      GGTAGATTAATTTAAAGCTCATTAAGATAGCATTTAAGT--ACTGGGAAGGATTGATGGG
GCATGGGCAGACCTCCACCATAAGCTAGCTAC      GGTAGATTAATTTAAAGCTCATTAAATAGCA--TTTAAT--ACTGGGAAGGATTGATGGG
                                                *                                 *       *

TTATTGTTGGCCTTCAGGCTAGGGAAGGCCCA      C-----------------------------------------------------------
TCCTTTGATGGCCTCCTGCTATGGAGGCCCAC      ACC---------------------------------------------------------
CCCCGTGGGGTCCTCGTGGATCCGGCTATGGA      -----------------------------------------------------------
GGCGTAAGCGCATTGCACTCTTGGGATTCCTG      CTATATTGAGGCTATTTC-----AATGACTTGCTACACTTGATGTGCCTTCTGCGCCGT-
GACGGTAAGCGCATGCCTCTTGGGATTCCTGA      CTATATTGAGGCTATTTC-----AATGACTTGCTACAGCTTGTGTGCCTTCTGCGCCTT-
GATCAGTCGTCTATACGACCTGCTGAACTAGA      CTCAA---AGACTATATCTGAGGTGTGCCTTATAATT--TATTATGTCTTG-----CCTT
GCATTCAGTCGTCTATACGACCTGCATGAACA      CTCAA---AGTCTATATCTGAGGTGTGCCTTATAATTTATTTATTGTCTTG----CCTTT
GGATCATGTCGTCTATACGACCTGCTGAACTA      CTCA----AGTCTATATCTGAGGTGTGCCTTATAATT--TTTTTGTCTTG-----CCTT
CCAATAGCAGACCTCCACCAATAAGCTAGCTA      CCTTAAGCAAACTGTATC-----TTTGCCTTAT-ATTGGCTCTGAACCTCAAAAGTCTT-
GCATGGGCAGACCTCCACCATAAGCTAGCTAC      CCTTAAC-AAACTGTATC-----TTTGCCTTATTATT--------GGCTCGTGCCACCTC
```



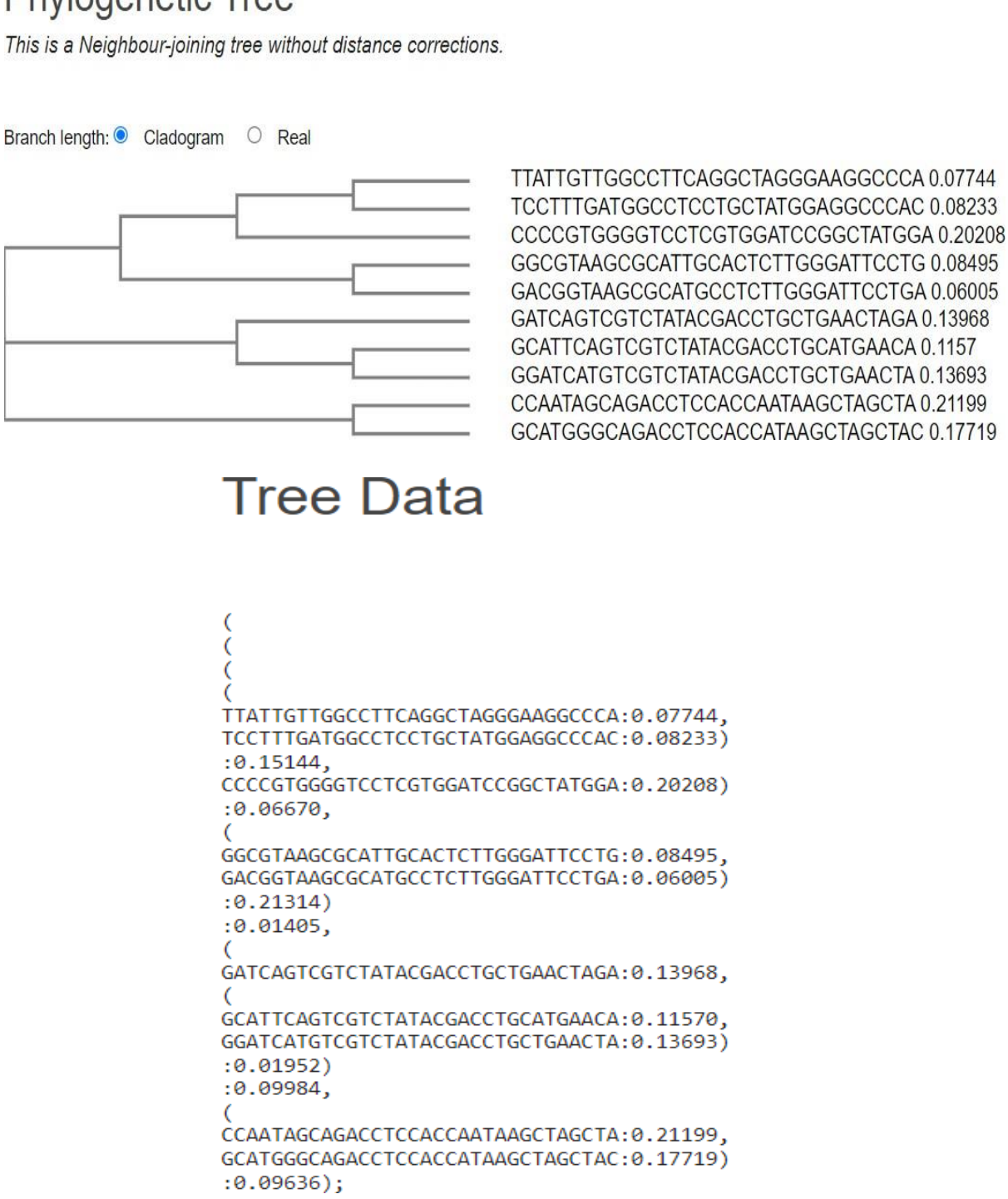


Figure 44: Phylogenetic tree established between all clades of coral species to find how one species is related to another

CHAPTER 8: RESULT

Average zooxanthellae density count for Siderastrea species is 3.69×10^6 cells/cm^2

Average zooxanthellae density count for Favites species is 3.45×10^6 cells/cm^2

Average zooxanthellae density count for Porites species is 3.66×10^6 cells/cm^2

The density of zooxanthellae cells showed strong negative correlations with light intensity and a weak inverse correlation between temperature.

The PCR product obtained from the samples was sequenced to explore further the phylogenetic position of the zooxanthellae in the Gulf of Kachchh. The phylogenetic positions revealed on the relationship between each species by distance methods.

The sequencing result showed that there are three symbiodinium species that is identified. The Genus Siderastrea has two species which are further divided into sub-species that are distinctly distant and not related to each other. The distance calculated between the sub-species using distance method and maximum likelihood methods helps to tell that the individual sub-species are close to one another by a mere 0.01 to 0.2 difference. The Genus Favites has two branching of species, out of which one is sub-divided having a sub-species. The distance between them is 0.02 difference meaning the species are although diverged from their origin and is a part of the divergent evolution but, the cladogram represents the distance is not that much. The Genus Porites has only two species and is differentiated by a distance of 0.1 difference.

CHAPTER 9: DISCUSSION

The experiment conducted for the study of symbiodinium cells under severe stress condition helped us to focus light and understanding in its association with the coral. The isolation of zooxanthellae from each od the coral species was an extremely tedious task to perform and required a lot of time. In addition to that, after isolation, grinding of the tissue to make a fine slurry with mortar and pestle is best to obtain maxium yield and DNA content from individual species. The use of WaterPik device was also useful in the extraction process but, proved to not provide with good amount of DNA yield as compared to the traditional method of mortar and pestle for DNA isolation. The Qiagen Plant Mini kit for further isolation of the DNA versus the traditional method was both effective as extraction were performed using both to understand better efficacy of specific methods. Few times adequate DNA wasn't collected and experiments were repeated and performed in triplicates/duplicates for more than 7-8 times to achieve the best amount the DNA for amplification. During the PCR amplification, amplification events were carried out for a total of six times for desired amplicons. After achieving the amplicon product, it was visually qualified by running on a gel unit for thrice and then checking for its quantity with the help of Nanodrop spectrophotometer. If dilution required before purification of the product, then it is done by diluting the samples with DNase-free, RNase-free, nuclease-free by prior calculation in advance. Purification steps involved loading the maximum amount of amplicons to the gel and after a successful run, excise of the gel from the gel using a sharp scalpel, followed by the Qiagen purification kit for PCR cleanup. After the cleanup of the PCR products, each coral species was sent for whole genome sequencing. The analysis of the sequencing data obtained was also performed to establish relationship between species and how they are related in a phylogenetic tree. Finally, phylogenetic tree was obtained using bioinformatics software. With the help of advanced knowledge of the bioinformatics databases more sequencing work could have been generated and if time was not a constrain then, pigment analysis of the chlorophyll compounds present in the symbiodinium responsible for photosynthesis could also be performed by incorporating the use of fluorometer and high-performance liquid chromatography techniques. Nevertheless, physiological, culturing, and molecular identification related study of the symbiodinium cells associated with the corals of Gulf of Kachchh provided us with deep insight into the field of marine biology.

CHAPTER 10: SUMMARY AND CONCLUSION

A variety of commonly used methods for the separation of coral tissue from its underlying skeleton were attempted. Razor-blade scrapes were found to be the most successful in terms of efficiency both in the low volume of sample produced as well as a dramatic reduction in handling time resulting in consistent good quality DNA extraction. The WaterPik method produced high volumes of extract resulting in lower yields of the sample during the concentration process. The air-brush technique reduced the volume of the samples considerably but concentration of the sample was still required. Disruption of the tissue and skeleton using a mortar and pestle was also found to result in smearing of the DNA when visualized on an agarose gel possibly due to mechanical degradation due to the presence of the calcium carbonate skeleton. The host DNA was found to be particularly sensitive to excessive handling such as repeated wash techniques whereas the dinoflagellate algal component was found to be more resistant to DNA extraction. All three extraction buffers that were used during the separation of the algal and invertebrate components resulted in similar DNA yields for the symbiotic algae. Using 100 mM EDTA and 10 mM Tris resulted in lower host DNA yields biotic algal component, in the pellet, contains a small fraction of contaminating animal host DNA. Deoxyribonuclease could be added to digest any host DNA present; however, prior to the lysis of the algal cells for DNA extraction, complete removal of the deoxyribonuclease has to be ensured to prevent loss of algal DNA. This technique was not used in this protocol but rather an SDS wash was used to remove the animal host. The supernatant contained mostly animal tissue with very few algal cells. As a percentage of the initial slurry there were 0.72% algal cells indicating that the supernatant samples were host-enriched. It is possible that within the host sample there is some contamination of the coral DNA with DNA from Symbiodinium because of autolysis during the separation process. This source of contamination of coral DNA is considered to be minimal due to the fact that Symbiodinium cells are difficult to lyse without enzymatic or high-pressure treatment and because there was no evidence of lysed cells during cell counts. After the separation procedure, the algal counts are slightly lower (81%) than the initial counts. Centrifugation of the separated cells results in two pellets, a white one containing host debris such as nematocysts, calcium carbonate and mesoglea and a dark brown pellet that contains the symbiotic algae. On resuspension of the Symbiodinium, in an attempt to reduce contamination from host tissue, the cells at the border with the white pellet were not incorporated into the assay.

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