BIOCHEMICAL PROPERTIES OF ANEMONE FISH MUCOUS TO UNDERSTAND ITS ADAPTATION TO SEA ANEMONE

BY

NAJATUL SU AD BINTI ABDULLAH

A thesis submitted in fulfilment of the requirement for the degree of Master of Science (Biosciences)

Kulliyyah of Science
International Islamic University Malaysia

JANUARY 2016

ABSTRACT

The study of anemone fish mucous to understand anemone fish adaptation to sea anemone was aimed to identify the biochemical composition in the fish mucous that enabled the protection of anemone fish against the stinging tentacles of sea anemones. Upon sampling, one species of sea anemone, *Heteractis magnifica* was identified with its resident anemone fish, the false clownfish, *Amphiprion ocellaris,* along with 2 other non-symbiont coral reef fish species, the scissor-tailed sergeant, *Abudefduf sexfasciatus* (Family Pomacentridae) and the moon wrasse, *Thalassoma lunare* (Family Labridae) to provide comparison and knowledge insight into anemone fish ability to live unharmed along the tentacles of sea anemone, *H. magnifica*. The scissor-tailed sergeant and the moon wrasse are among many other coral reef fishes that are observed to be stung upon contact with the sea anemone tentacles that elicit nematocyst discharge. Mucous of false clownfish, *A. ocellaris* was extracted and investigated for its glycoprotein by studying the protein and sialic acid content and compared against the mucous content of sea anemone, *H. magnifica*, and other fish species, the scissor-tailed sergeant, *A. sexfasciatus* and the moon wrasse, *T. lunare*. Protein identification was done by SDS-PAGE and MALDI-TOF analysis while thiobarbituric acid assay followed by HPLC detection were performed to assess sialic acid content in the mucous. Results indicated that the false clownfish had keratin and actin glycoproteins mucous with 1.636 mg/ml sialic acid content while for non-symbiont fish, the scissor-tailed sergeant presented collagen glycoprotein with 50.433 mg/ml concentration of sialic acid and moon wrasse had high molecular weight proteins with 71.893 mg/ml sialic acid content. The higher concentration of sialic acid was involved in causing the tentacles of sea anemone to discharge toxin. Hence, it is concluded that the false clownfish could afford protection from the stinging toxins of sea anemone by having glycoproteins with very low content of sialic acid which was not adequate to trigger sea anemone response for toxin discharge.

خلاصة البحث

إن دراسة مخاط سمكة المهرج لإدراك قابلية التكيف سمكة المهرج لشقائق البحر تهدف إلى تحديد تركيبة كيميائية حيوية لمخاط سمكة المهرج الذي يحميها من لسعة مجس شقائق البحر. أثناء الاختبار، تُستخدم إحدى شقائق البحر، هيتيراكتيس ماغنيفيكا *Heteractis magnifica*, وسمكة المهرج المقِيمة بها، أوسيلاريس أمفيبريون *Amphiprion ocellaris*، مع نوعين من السمكة المرجانية غير متعايشتين، الرقيب سسيسورتيل scissortail sergeant، أبو دفدوف سيكسفاسسياتوس *Abudefduf sexfasciatus* (فصيلة بوماسينتريداي Pomacentridae) ومُن الراس moon wrasse، تلاسوما لوناري *Thalassoma lunare* (فصيلة اللبروسية Labridae) مع كونها دراسة مقارنة وإلمام تام بقدرة سمكة المهرج على العيش سالمات بين مجس شقائق البحر هيتيراكتيس ماغنيفيكا *H. magnifica*. بطبيعة الحال، يلاحظ أن الرقيب سسيسورتيل scissortail sergeant ومُن الراس moon wrasse من بين الأسماك المرجانية الأكثر ملدوغة عند الاحتكاك بمجس شقائق البحر التي تنتزع كيسة لاسعة. يتم البحث باستخراج مخاط أوسيلاريس أمفيبريون *A. ocellaris* وفحص بروتينها السكري بطريقة دراسة مضمون البروتين وحمض السياليك ومقارنته بمضمون مخاط شقائق البحر، هيتيراكتيس ماغنيفيكا *H. magnifica* وكذلك مضمون السمكتين المرجانيتين الرقيب سسيسورتيل scissortail sergeant، أبو دفدوف سيكسفاسسياتوس *A. sexfasciatus* ومُن الراس moon wrasse، تلاسوما لوناري *T. lunare*. وأجرى فحص برادفورد للبروتين Bradford assayوالفصل الكهربائي لهلام كبريتات دوديكل الصوديوم متعددالأكريلامايد SDS-PAGE في تحقيق البروتين، ومن ثم أجرى اختبار حمض ثيوباربيتورات وعملية كروماتوغرافيا سائلة عالية الأداء HPLCلتقييم مضمون حمض السياليك لخلاصات المخاط. وتشير النتيجة إلى أن سمكة المهرج لها وزن جزيئي منخفض وقلة مضمون حمض السياليك. وأما السمكتان المرجانيتان غير متعايشتان تتميزان بوزن جزيئي مرتفع وكثرة مضمون حمض السياليك. فمن هنا يتضح أن حمض السياليك يسبب نزع مجس شقائق البحر ذيفانها. ومن هنا، نستنتج بأن سمكة المهرج قادرة على حماية نفسها ضد لسعة مجس شقائق البحر المسمومة إِثْرَ تضمنها بروتين السكري دون حمض السياليك.

APPROVAL PAGE

I certify that I have supervised and read this study and that in my opinion, it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Master of Science (Biosciences).

…………………………………..
Shahbudin Saad
Main Supervisor

…………………………………..
Deny Susanti
Co-Supervisor

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Master of Science (Biosciences).

…………………………………..
Tengku Haziyamin Tengku Abd. Hamid
Examiner

This thesis was submitted to the Department of Biotechnology and is accepted as a partial fulfilment of the requirements for the degree of Master of Science (Biosciences).

…………………………………..
Suhaila Mohd Omar
Head,
Department of Biotechnology

This thesis was submitted to the Kulliyyah of Science and is accepted as a fulfilment of the requirements for the degree of Master of Science (Biosciences).

....
Kamaruzzaman Yunus
Dean, Kulliyyah of Science

DECLARATION

I hereby declare that this thesis is the result of my own investigations, except where otherwise stated. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at IIUM or other institutions.

Najatul Su Ad Binti Abdullah

Signature .... Date ....

INTERNATIONAL ISLAMIC UNIVERSITY MALAYSIA

DECLARATION OF COPYRIGHT AND AFFIRMATION OF FAIR USE OF UNPUBLISHED RESEARCH

BIOCHEMICAL PROPERTIES OF ANEMONE FISH MUCOUS TO UNDERSTAND ITS ADAPTATION TO SEA ANEMONE

I declare that the copyright holder of this thesis is International Islamic University Malaysia.

By signing this form, I acknowledged that I have read and understand the IIUM Intellectual Property Right and Commercialization policy.

Affirmed by Najatul Su Ad Binti Abdullah.

……………………………. ………………..

Signature Date

ACKNOWLEDGEMENTS

All Praises to Allah, Most Gracious and Most Merciful. This thesis was possible with the supervision of Assoc. Prof. Dr. Shahbudin Sa'ad and Assoc. Prof. Dr. Deny Susanti, to whom both I owe my lifetime of gratitude for their guidance, patience and encouragements throughout the research.

I would like to acknowledge the scholarship of IIUM for my appointment as an Academic Trainee. Thank you to all staff of Kulliyyah of Science and friends that helped me greatly in my research works and study period here including Br. Azizul Aziz, Br. Muhammad Faiz, Miss Nasihin Seth, Br. Amir, Mrs. Fikriah Faudzi, Br. Fuad Miskon, Sr. Juliana, Br. Zaini, Br. Fikri Akmal, Br. Hamizan, Br. Husaini, Miss Nor Shuhadah Warzukni, Br. Muzzamil, Miss Aminah Kaida, Miss Hafizah Zakaria, Miss Amira Farzana, Br. Hendy Putra, Br. Jazli Aziz, Br. Fuad Anuar, Miss Amalina Soudin and many more.

I would also like to express my heartfelt apologies and appreciation to my family; my mum, Mrs Faridah Hamdan, my dad, Mr Abdullah Sadli, my in-laws, Mr Mohd Sidek and Mrs Zawiah, my darling husband, Mr. Ahmad Mustaqim, and my baby boy who grew with this thesis, Ahmad Amsyar, for giving me strength when I felt weak and believed that I could accomplish this. Last but not least, my daughter, Mawar Hani, whom I delivered with this thesis. Thank you.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF THE STUDY

Anemone fishes are brightly coloured coral reef fishes classified under the damselfish group (Family Pomacentridae) and named such for its association with sea anemone. This association have been studied for various aspects such as occurrences and natural habitat since it was first reported observed in nature by Dr. Cuthbert Collingwood in 1868 (Randall & Fautin, 2002). The fish habitually swims toward the tentacles of sea anemone and appears unperturbed though other organisms or fishes are stung upon adherence of the sea anemone tentacles. This lies the uniqueness of the symbiosis between the anemone fish and sea anemone.

Initially only anemone fish was thought to benefit from the symbiotic relationship with sea anemone host by protection from predation and as a nesting ground but research into the field has shown that the sea anemone host also benefits from the presence of the anemone fish. It has been documented that anemone fish does oxygenate its host (Szczebak, Henry, Al-Horani, & Chadwick, 2013) while provides nutrient and water circulation to encourage growth (Holbrook & Schmitt, 2004).

Recent research on anemone fish symbiosis focus on its physiological make up, genetic divergence of the anemone fish species, geographical distribution, its host imprinting and biochemistry relationship between the fish and its host. This indicated that many still query how the symbiosis is enabled. Many researches revolved around the question whether the protection of anemone fish from the toxins of sea anemone is naturally innate or could be acquired through acclimation process, though a generally accepted opinion held that the fish could have afford protection from the stinging nematocysts by a biochemical constitution in their mucous layer.

Previously Lubbock (1980) showed that one species of anemone fish, the Clark clownfish, *Amphiprion clarkii* (Bennett, 1830) mucous layer appeared to be three to four times thicker than that of related fishes that do not inhabit anemones and consists largely of glycoprotein. Lubbock (1981) further tested biological compounds to a sea anemone species that host *A. clarkii*, the *Stichodactyla haddoni*. He reported that protein and glycoprotein elicited strong response while polysaccharide and lipid gave weaker response. However, no other research on other species of anemone fishes was done to enable generalization of anemone fishes mucous composition or any other compound responsible for the protection.

The uniqueness of the symbiotic relationship is further heighten by the knowledge that only 10 species of sea anemone is reported to support anemone fish presence while there are more than 800 species of sea anemone discovered worldwide (Roopin & Chadwick, 2009). Sea anemones are classified under order Actiniaria, class Anthozoa, phylum Cnidaria, which characteristically possess nematocyst that release toxins when triggered. Toxins from various sea anemone species known to host anemone fish and non-host have been elucidated. Significance of these discoveries lead to enhancement of medicinal therapies

Additionally, the geographical distribution of anemone fishes and sea anemones hosting symbiont fish is abundant in coastal waters surrounding Malaysia. Even though sea anemones are found worldwide only the sea anemones found in tropical waters host anemone fish. Malaysia’s tropical seas is situated in the convergence of Andaman Sea, South China Sea, and Sulawesi Sea, which are the boundaries between the Indian Ocean and the Pacific Ocean. That puts Malaysian waters in the Coral Triangle area which although includes countries such as Philippines and Indonesia, it is an area with the world's highest marine biodiversity.

Its marine area which spans 351,000 km^2 (Spalding, Ravilious, and Green, 2001), with 3,600 km^2 coral reef area, more than 350 species of coral identified and to an approximate of 900 coral reef fish species, which boost prominent fish groups as damselfishes, anemone fishes, labrids, butterflyfishes, groupers, moray eels and even the elusive triggerfish. Malaysia nurtures the richest biodiversity of coral reefs, seagrass beds, mangrove forests and other important habitats in the world making it beneficial as the research area and may further enhance Malaysia's recognition in natural marine resources.

Due to the lack of literature on the isolation of chemical compound from anemone fish that is directly involved in its host symbiosis, this project aims to find the compound that may contribute towards further comprehension of co-existence of anemone fishes with sea anemones. The coasts of Malaysia have high anemone fish species richness and also a globally valuable area of fish biodiversity, thus provide a good research site for this project.

In this study, one species of sea anemone hosting anemone fish, one species of its symbiont anemone fish, and two non-symbiont fish species were identified during sampling collection. Mucous extracts obtained from the sea anemone and three species of fish were investigated by the composition of glycoprotein through Bradford assay, SDS-PAGE and MALDI-TOF techniques for protein identification and thiobarbituric acid assay followed by HPLC detection for sialic acid determination. The two non-symbiont fish species identified in the Family Pomacentridae and Family Labridae were used as comparison throughout the test. Ichthyotoxicity test by which the survival of the fish against host anemone toxin was observed and also sea anemone response to sialic acid was carried out.

1.1.1 Benefit of Study

Anemone fishes have been the interest of many, researchers, divers and nature lovers alike. Increasing the knowledge gap in the anemone fishes research is one of the many benefits of this study. Details on anemone fish mucous provide basic information for further characterisation of the fish. With the current advancement in scientific research, previous knowledge on anemone fishes was assayed with new techniques. This study was imperative as the species originated in Malaysia coastal waters were different than those found in literature

1.2 RESEARCH QUESTION

How does the protein from the anemone fish mucous differ from other coral reef fish that enables protection from sea anemone stinging?

1.3 RESEARCH HYPOTHESIS

The mucous coat of anemone fish contains a protein with a different composition specifically the sialic acid, a biochemical compound that protects the fish against the toxin sting of sea anemones.

1.4 RESEARCH OBJECTIVE

1.4.1 General Objective

To determine the protein and sialic acid component in the anemone fish mucous coat glycoprotein and to understand how the anemone fish in symbiosis with sea anemone is protected against stinging.

1.4.2 Specific Objectives

1. To identify selected sea anemone species, anemone fish species and non-symbiont fish species.
2. To detect and determine the protein content from the mucous of *Amphiprion* sp., other non-symbiont fish species and sea anemone
3. To determine and compare the sialic acid content from the mucous of *Amphiprion* sp., other non-symbiont fish species and sea anemone.

CHAPTER TWO

LITERATURE REVIEW

2.1 ANEMONE FISH BACKGROUND STUDY

Anemone fishes are among the many brightly coloured fishes of the tropical coral reef habitat that fascinates the marine environment. Anemone fishes are classified under phylum Chordata for animals with tissue, coelom, dorsal, tubular nerve cord, and post anal tail, class Actinopterygii ray-finned spiny fish, order Perciformes for oval shaped body laterally compressed, and family Pomacentridae for damselfishes.

Anemone fishes are easily recognisable by the striking colour patterns. For example, the three anemone fishes, *Amphiprion ocellaris, A. percula* and *A. clarkii*, have the characteristically famous orange white vertical bands. Differences between the three species can be identified by other features such as the size of the bands, with black markings, and number of spines in their dorsal and anal fin. Knowledge on their geographical distribution also assists in species identification as existence of *A. ocellaris* with its nearest species sibling, *A. percula* do not overlap (Timm, 2008).

Anemone fishes are also recognisable by their distinctive behaviour swimming near sea anemones and retreating into the sea anemone tentacles upon caution of danger or a predator attempts to attack. This lays the uniqueness of the anemone fish as it is unharmed or appears undisturbed by the tentacles of the sea anemones because the tentacles are known to possess stinging capsules, nematocyst, that discharges toxin to prey or predator that approaches near the sea anemone.

Many researches have marvelled the anemone fish ability to live among the sea anemone. Some have isolated and characterized the toxins of various sea anemones, host and non-host to fish, as part to understand this symbiosis. Sea anemones are generally found in marine environments around the world but due to the geographical distribution of anemone fishes in tropical coastal water regions, only 10 species of sea anemones are known to host fish (Fautin & Allen, 1992).

Other researchers studied on the anemone fishes, its genetic diversity (Timm, 2008), its chemical biology (Mebs, 2009), its behaviour in response to host (Elliott & Mariscal, 1997) among many strategies to document and explain the mechanism by which the anemone fish adapts to sea anemones. The mostly applicable opinion is that these fish have a biochemical composition in their mucous layer that provides protection from the toxins or stinging tentacles.

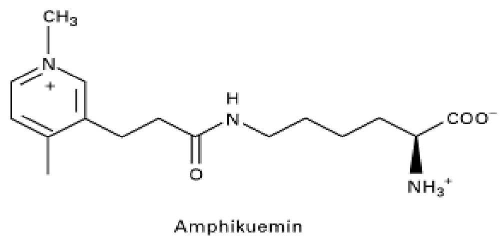
However, the conflicting opinions are on the source of the protective mucous coating whether it is inborn or assimilated. To date, two hypotheses are generally believed by which the symbiosis may occur. Either the fish essentially produces its own mucous coat with biochemical compounds to protect itself from the stinging toxins of the sea anemone tentacles or otherwise, the fish alters the biochemical compounds in its mucous coat after a behavioural process by which the fish acclimatised to the host sea anemones.

Lubbock in 1980 tried to research on the base of the first hypothesis that Clark's anemone fish, *A. clarkii* mucous contained the answer to the mechanism that enable the symbiont-host relationship. He studied the thickness of the mucous, estimated protein by electrophoresis and used *Stichodactyla haddoni* for radiolabelling of nutrient transfer. He concluded that though the fish mucous appears thicker than other non-symbiotic fish and traces of the sea anemone mucous is found in the mucous coat of the fish, *A. clarkii* is protected from stinging by its external mucous layer that is not fundamentally dependent upon the host.

Lubbock (1981) further quoted Schlicter (1976) who used radiochemicals and isoelectric focusing with "inhibitory substances are produced by anemone and surface of the fish do not have protecting substance". This raises the question whether the fish contained substances to protect itself against the toxin or its mucous mimics the sea anemones mucous to not induce nematocyst discharge. Different groups of compound were tested and Lubbock (1981) reported that protein and glycoprotein elicited strong response from *S. haddoni* while polysaccharide and lipid stimulated the nematocyst less. However, the nature of the substances in the fish mucous that cause or inhibit response remains unclear.

On the second hypothesis, Brooks and Mariscal (1984) attempted to understand the acclimation process by using rubber band fashioned as tentacles attached to rock as surrogate anemone. They cited works of Martin in 1968 who reported that fish pick up species specific antigen of anemone and Foster in 1975 who reported that fish produce protein after acclimation which was not present in non-acclimated fish or anemone toxin. They concluded their findings by which the anemone fish alters the mucous coat during acclimation but the presence of surrogate anemone decreases acclimation.

Elliot and Mariscal (1997) further studied acclimation and the process of acquired protection of three selected species of anemone fishes against three selected sea anemone species which were randomized to be natural host or unnatural host to selective fish species. Only one fish species, *A. clarkii* was not stung by initial contact to all sea anemone tentacles. Another species, *A. ocellaris* was stung by tentacles of *Heteractis crispa* and *Macrodactyla dorensis*, as these two sea anemone were unnatural host to *A. ocellaris*. For *A. perideraion* (Bleeker, 1855), adhesion and protection were mixed from its natural and unnatural host species. Hence, to indicate that naive anemone fish is innately protected can be misleading, as through experiments conducted by many, including Miyagawa (1989) suggested that chemical cues enable the symbiosis process. Miyagawa (1989) published the isolation of amphekuemin (1), a pyridium alkaloid, that appealed swimming of *A. perideraion* fish towards its expected sea anemone host, *Radianthus kuekenthali*.



(1)

It might be possible that more study on the variation of anemone fish species mucous against selected host sea anemone species may provide knowledge to further understand the protection mechanism and symbiosis adaptations.

2.1.1 False Clownfish

The False Clownfish, *A. ocellaris* (Cuvier in Cuvier and Valenciennes, 1830) is a popularly photographed species of anemone fish in the coastal waters of peninsular Malaysia and the coral triangle region. It can be found in shallow water reefs of 1-15 m depths waters because of their association to sea anemone which requires penetration of sunlight for photosynthesis. It is easily recognised by its orange and white bands with black linings on its small body and habitually swimming near or into the sea anemone tentacles of the coral reefs (Timm, 2008).

Figure 2.1. A photograph of a common false clownfish in Malaysian waters contributed by Muhammad Faiz, Department of Marine Science, IIUM.

Its geographical distribution range from the tropical region that include countries of Indo-West Pacific; Indian Ocean to Andaman and Nicobar Islands, Red Sea, South East Asia region, Thailand, Malaysia, Indonesia, and the Philippines; and northwest Australia to Singapore, and the Western Pacific to Taiwan and the Ryukyu Islands (Ferrari Andrea and Ferrari Antonella, 2006). Their limited distribution is due to its short larval stage resulting in limited larval dispersal.

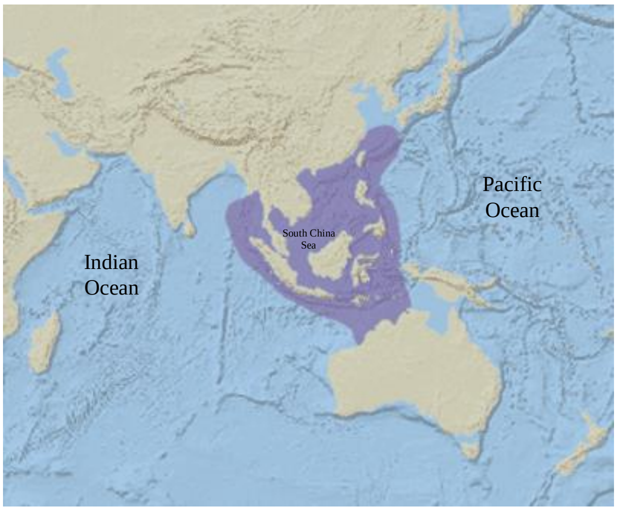


Figure 2.2. Geographical distribution of false clownfish population. Retrieved September 15, 2013, from FishBase website: http://www.fishbase.org/summary/6509.

The false clownfish is categorised as a host specialist contrary to the Clark's anemone fish, *A. clarkii* which is categorised as a host generalist. Host specialist means the fish, *A. ocellaris* only adapts to specific species of sea anemone, namely, *H. magnifica, S. gigantea,* and *S. mertensii*. Host generalist fish like *A. clarkii* may live mutually to almost all species of host sea anemone. The dependency of the fish to the host might vary due to their swimming capabilities. Poor swimmers such as *A. ocellaris* maintain close distance to its host while efficient swimmers may wonder far and retreat quickly upon danger in a loose association manner.

The false clownfish adheres to strict linear dominance hierarchy, living in small structure groups in each host sea anemone. They are sexually dimorphic with the size of the female larger than the male fish. The largest fish in the hierarchy of one host sea anemone is the dominating female fish and the second largest is the dominant male which is also the breeder male in the group (Michael, 2008).

The distinct pair is monogamous throughout the lunar spawning period. The dominant female will breed with only the dominant male fish by which the female releases 100 to 1000 eggs fertilised by the male. Once hatched, the juvenile fill in the ranks after the dominant male fish as non-breeder subordinates by size, largest to smallest, which upon maturity or over crowdedness would find other host sea anemone to inhabit. As non-breeder subordinates, the juveniles or sub-adults demonstrate psychophysical castration which the development of mature sex organ and growth are repressed (Fautin & Allen, 1992).

2.2 SYMBIOSIS WITH SEA ANEMONE

Symbiosis is defined as the living of two organisms closely together or relying on one another in a habitat or ecosystem for the benefit of both of the organisms. It differs from parasitism which only benefits one and harms another or commensalism which benefit one party but does not help the other (Michael, 2008). In symbiotic relationship, both organism will benefit mutually.

Mariscal (1970) reviewed reports of previous research observations to conclude the nature of the anemone fish symbiosis to its host sea anemone. He denoted that both benefit mutually by this relationship even though sea anemone may be observed surviving in the wild without hosting fish. The fish are obligate symbiont that benefit primarily by protection from predator, nesting and breeding grounds, parasite removal, tactile stimulation and possibly eating the sea anemone as some food source.

On the other hand the sea anemone may benefit more as they also receive protection from predator as the symbiont fish may help deter away and afford tactile stimulation. As the fish swim around the tentacles, the fish may contribute to remove parasites and necrotic tissues and organic and inorganic materials from the oral disc or sea anemone along with providing the host with water and food circulation.

This is later proven as Szczebak et al. (2013) investigated on the anemonefish movements and its effect on oxygenating the sea anemone host. Roopin, Henry and Chadwick (2008) demonstrated nutrient transfer from the fish to the sea anemone. Furthermore, studies found sea anemone with removed resident fish tend to die off. This could also be due to predation of butterflyfish which come to graze on the polyps of sea anemone when there are no anemonefish to chase them away.

Sea anemone are distributed worldwide having the greatest species diversity at 30 – 40 ° N and S. According to Fautin and Allen (1992), the 10 species of anemones that host anemone fish are taxonomically diverse. These belong to five genera from three families: family Stichodactylidae which includes *S. haddoni, S. gigantea, S. mertensii, H. magnifica, H. crispa, H. aurora,* and *H. malu*; family Actiniidae species