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Studio di genetica di popolazione di *Ceraesignum maximum* (G. B. Sowerby I, 1825) (Mollusca, Gastropoda) lungo le coste di Okinawa (Giappone)

A study of population genetics of *Ceraesignum maximum* (G. B. Sowerby I, 1825) (Mollusca, Gastropoda) along coasts of Okinawa (Japan)

Tesi di Laurea Magistrale di:

Eleonora Negro

Relatore:

Prof. Carlo Cerrano

Correlatore:

Prof. James Davis Reimer

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RIASSUNTO

L’arcipelago delle Ryukyus si trova situato tra il sud del Giappone e Taiwan, di cui l’isola principale è Okinawa. La particolarità di quest’area è la presenza della corrente di Kuroshio, la quale scorrendo da sud verso nord permette con le sue acque calde la presenza di coralli a latitudini tra i 24 e i 30°N (Kayanne et al., 2004) e ricopre un ruolo chiave nel favorire l'alta ricchezza di specie della fauna corallina in Giappone (Nishihira, 2004). Inoltre, la ricchezza di specie in queste isole è elevata e diminuisce muovendosi verso nord.

In particolare, le acque cristalline di Okinawa fanno da sfondo a più di 340 specie di coralli (Nishihira e Veron, 1995; Nishihira, 2004). Sfortunatamente, negli ultimi anni, a causa di diversi fattori sia antropogenici che biotici, oltre alle barriere coralline di Okinawa anche quelle delle Isole Ryukyus hanno subito un degrado (Sakai e Nishihira, 1986; Mori, 1995; Omori, 2011) e si ipotizza che una delle cause naturali con effetti negativi sui coralli sia da ricercarsi nel gasteropode vermetidae *Ceraesignum maximum* (G.B.Sowerby I, 1825) (Shima et al. 2010, 2013).

Questo mollusco è ampiamente distribuito nelle barriere coralline dell’Indo-Pacifico (Hadfield et al., 1972; Hughes and Lewis, 1974; Zvuloni et al., 2008) e nel Mar Rosso (Hughes e Lewis 1974; Kappner, 2000; Brown, 2018).

Le particolarità di questo organismo sono di avere una conchiglia tubulare che cresce all'interno della matrice scheletrica dei coralli o su substrato inerte (Morton, 1955; Keen, 1961; Savazzi, 1996; Rawlings et al., 2010; Bieler & Petit, 2011; Golding et al., 2014) e di utilizzare reti di muco come strategia alimentare (Morton, 1965; Hughes and Lewis, 1974; Kappner, 2000).

Le caratteristiche ecologiche di *C. maximum* sono causa di effetti negativi nei coralli, le cui alte densità e probabilmente anche le reti di muco, possono causare una riduzione dello sviluppo scheletrico dei coralli fino all'81% e una diminuzione della sopravvivenza dei coralli fino al 52% (Shima et al., 2010).

Ciononostante, diversi aspetti di *Ceraesignum maximum* sono ancora sconosciuti e altri necessitano di ulteriori osservazioni, soprattutto per quanto riguarda la genetica di popolazione. Lo scopo di questo studio è quello di attestare la diversità genetica delle popolazioni di *C. maximum* in particolare concentrandosi sulla differenziazione tra la costa occidentale e quella orientale di Okinawa. In aggiunta, un altro confronto è stato realizzato per verificare una possibile variazione tra le popolazioni localizzate in siti con la presenza di impatto antropogenico con quelli naturali.

Lo svolgimento dello studio ha previsto il campionamento degli esemplari in diversi siti lungo le coste di Okinawa, l'estrazione e amplificazione del DNA mitocondriale cytochrome C oxidase subunit I (COI mtDNA) e le successive

analisi per l'individuazione degli aplotipi e visualizzarne il loro pattern e effettuare l'AMOVA.

I risultati ottenuti hanno mostrato che *C. maximum* costituisce una popolazione mista, non recente, la quale ha alleli unici; però sono necessari più campioni per poter avere una visione più completa.

Per quanto riguarda i dati dell'analisi statistica viene riportata che la maggiore variazione si ha all'interno delle sottopopolazioni nei vari siti e in aggiunta non è stata rilevata una differenza significativa nelle sottopopolazioni sia per il confronto tra le due coste sia tra siti impattati e non.

Nel complesso, i risultati ottenuti forniscono una prima baseline riguardante la genetica di popolazione di *C. maximum*, da integrare con approcci riguardanti la morfologia, l'ecologia e la distribuzione per definire con maggiore chiarezza molteplici aspetti di questi organismi che sono ancora relativamente sconosciuti, in particolare nella zona di Okinawa.

Chapter One

INTRODUCTION

1.1 Okinawa's coral reefs

The Ryukyus Islands (Fig 1) consist of a group of islands distributed between the southern part of Japan and Taiwan in the East China Sea. This archipelago is situated in a monsoon area and it is influenced by the Kuroshio Current (Kayanne et al., 2004) (Fig 1), which streams towards the north along the west side of the Ryukyus Islands (Andres et al., 2008). This current plays a major role in causing the high species richness of coral fauna in Japan (Nishihira, 2004) and allows the existence of coral reefs at the relatively high latitudes of 24° to 31°N (Kayanne et al., 2004). Another significant aspect of this warm marine stream, besides increasing the sea surface temperature (SST), is to supply coral larvae from the southern zone towards more northern areas, sustaining the coral reefs of the archipelago (Kayanne et al., 2004). Species richness has been shown to be at the highest in the coral reefs around the Ryukyus Islands and decreases moving north (Nishihira, 2004).

The Ryukyu Archipelago is divided into three groups: the Northern Ryukyus represented by the Tokara Archipelago; the Middle Ryukyus with Okinawa and

Amami Islands, and the Southern Ryukyus with Yaeyama and Miyako Islands (Nishihira, 2004). In the Middle Ryukyus rather large islands can be found, with rivers, that often host mangrove forests (Tsuchiya, 2004), and uneven coast lines which give life to different habitats in shallow water close to the shore (Nishihira, 2004).

Okinawa Island (Fig 1), or in Japanese Okinawa-Jima (26°05′N, 127°39′E–26°52′N, 128°15′E), is the principal and biggest island of the archipelago of the Ryukyus, with a length of 100 km and an area of 1208 km^2 (Hongo et al., 2013). Additionally, smaller islands, acknowledged for their relatively immaculate condition, can be found along its coasts (Reimer et al., 2019).

The geological formation of the island is attributable to volcanic and sedimentary activities, uplift phenomenon, and coral reef development (Kayanne et al., 2004; Fujita et al., 2015). Moreover, this area has strong tidal phenomenon, which can range from 25 cm during neap tides up to 2 m on spring tides (Tsuchiya, 2004), and the annual average SST is around 25.0 °C (Sakai, 2004).

In this island the vast majority of coral reefs are fringing and patch reefs although in the southeastern coast there is a barrier reef (Sakai, 2004). The crystal-clear waters of Okinawa host more than 340 species of scleractinian corals (Nishihira and Veron, 1995; Nishihira, 2004), which is three times higher

than that observed on the Great Barrier Reef at comparable latitude (Veron, 1993; Nishihira and Veron, 1995). It is important to take into consideration that more than 70% of Japanese marine biodiversity still remains unknown (Fujikura et al., 2010).

Unfortunately, over the past years, due to different factors both anthropogenic and biotic, not only the coral reefs of Okinawa, but also the reefs around the rest of the Ryukyus Islands have been suffering degradation (Sakai and Nishihira, 1986; Mori, 1995; Omori, 2011). In some studies, one of the biological threats to corals has been identified as the snail worm (Shima et al. 2010, 2013), *Ceraesignum maximum* (G.B.Sowerby I, 1825), formerly known as *Dendropoma maximum* (see Golding et al., 2014), which is often found with its shell embedded in corals.

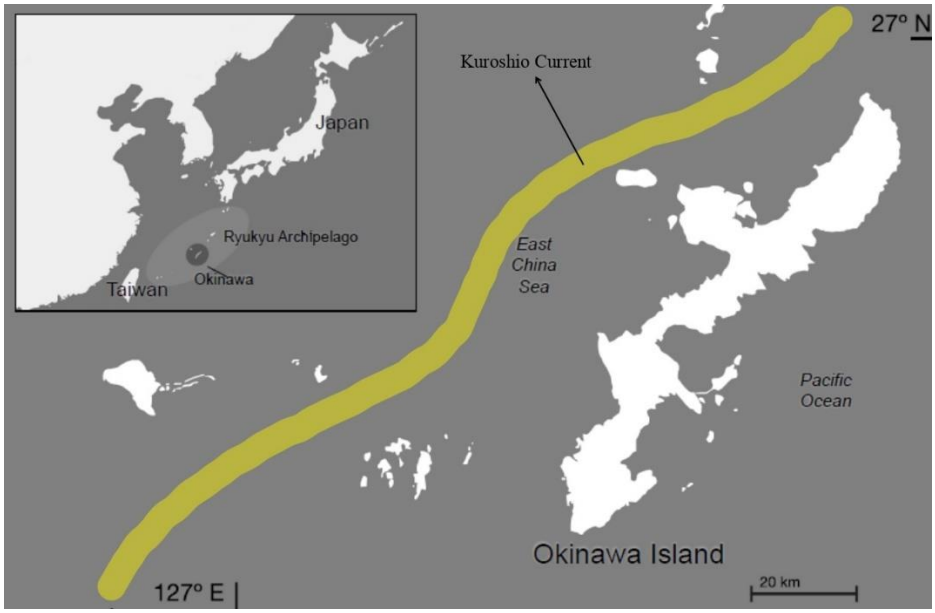

Figure 1. Archipelago of the Ryukyus, with a close up on Okinawa (modified from Masucci et al.,2019).

1.2 Corals' association with snail worms

Coral communities and especially scleractinian corals, with their structural complexity, play a significant role in creating habitats that can sustain a wide range of organisms (Gates & Ainsworth, 2011). The vast majority of individuals living their life associated to hard corals are invertebrates, with at least 869 species identified to belong to different phyla such as Arthropoda, Mollusca, Echinodermata, Annelida, Porifera, Platyhelminthes, Sipuncula and Hemichordata (Stella et al., 2011). However, a good number of species has not yet been described (Stella et al., 2011). Moreover, of the described species in Stella et al. (2011), 130 were mollusks and represented the second biggest component of organisms that live in association with corals. Some of these epibionts are sessile invertebrates such as vermetid gastropods, commonly known as snail worms, which have an uncoiled shell that can be partly or entirely overgrown and surrounded by the coral skeleton (Shima et al., 2013), and due to this growth form snail worms can cause morphological changes in their hosts (Bergsma, 2009; Shima et al., 2010).

1.2.1 Family Vermetidae

The subclass Caenogastropoda includes about the 60% of living gastropod species, of which a large portion is represented by marine families (Ponder et al, 2008).

One marine family is the Vermetidae, with at least 160 existing species of sessile suspension-feeding snails (Golding et al., 2014), and it is part of the Caenogastropoda clade. Vermetidae are characterized by uncoiled tubes-like shells, adapting to the substrate while growing (Morton, 1955; Keen, 1961; Savazzi, 1996; Rawlings et al., 2010; Bieler & Petit, 2011; Golding et al., 2014). These organisms are spread in tropical and temperate waters around the world (Safriel, 1974; Rawlings et al., 2010; Golding et al., 2014) and they are recognized for their role in creating habitats and their capability to interact with other sessile organisms (Golding et al., 2014). Another characteristic of many vermetid species is to have adopted as feeding strategy mucus nets that are extruded and retracted and which can trap suspended particles (Morton, 1965; Hughes and Lewis, 1974). Moreover, the difficulty of studying and therefore the lack of knowledge of this group of individuals is due to difficulty in their sampling, caused by their shell being embedded in the substrate (Bieler and Petit, 2011).

The largest vermetid gastropod species is *Ceraesignum maximum* (G.B.Sowerby I, 1825) (Hughes and Lewis, 1974; Shima et al., 2010; Phillips, 2011; Shima et al., 2013), which is the subject of this study.

1.2.1.1 Species *Ceraesignum maximum*

Ceraesignum maximum (Mollusca, Gastropoda, Vermetidae) (Fig. 3) is dominant and widespread in the coral reefs of the Indo-Pacific (Hadfield et al., 1972; Hughes and Lewis, 1974; Zvuloni et al., 2008), but its presence is also known from the Red Sea (Hughes and Lewis 1974; Kappner, 2000; Brown, 2018) (Fig. 2).

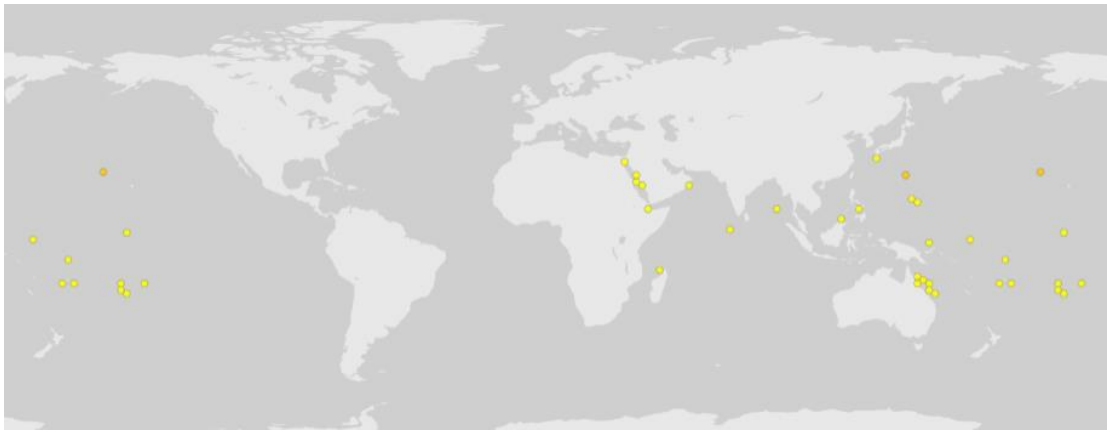

Figure 2. Distribution of *Ceraesignum maximum* (https://www.gbif.org/species/7927019)

Formerly, this species was placed in the genus *Dendropoma,* but Golding et al. (2014) in a systematic revision of the snail worm group identified this species as belonging to the genus *Ceraesignum*.

The molecular and morphological analyses conducted on *C. maximum* supports a close relationship with only one other species, the congener *Ceraesignum robinsoncrusoei*, and together these two species represent the only species belonging to the genus *Ceraesignum* (Golding et al., 2014).

The shell of *C. maximum* is not often visible since it is enveloped in the coral structure, but when embedded on dead substrata the shell is encrusted by turf and epifauna (Fig. 3: A, B, C, D, E, F, G and J), while the columellar surface and the interior of the shell are white (Golding et al., 2014).

Furthermore, the morphological description given by Golding et al. (2014) states that the body of the *C. maximum* snail worm is sturdy, and it is lodged in the last half of shell. The flat head is small compared to the foot muscle, and has a concave operculum attached that is made of chitin, and that can be covered by epifauna on the exterior, while the inner surface is smooth, except for muscle attachment area characterized by a “fingerprint” texture formed by concentric ridges of different heights (Golding et al., 2014).

The mouth presents a radula with up to 60 rows of teeth. The lateral tooth has one cusp on the inner side and five cusps on the external side, while the inner

marginal tooth has two cusps on the internal side and the outer side has three cusps (Golding et al., 2014). Moreover, the color of the head, the foot, the tentacles and the lips fade from cream to pale brown, while a dark black pigmentation is found in the sole, in the lateral surfaces of the foot, in the dorsal surface of head, with the exclusion of the tentacles, and opercular lobe. The frontal mantle margin it is trimmed with cream, then black bands and a shade of dark brown characterize the digestive gland, while the hypobranchial gland has a light yellow color (Golding et al., 2014). (Fig. 3K)

In this organism the rectum goes from the coelom to the frontal edge of the mantle cavity and the fecal pellets have cigar-shaped form (Golding et al., 2014).

Figure 3. (A, B, C, D, E, F and G) corals hosting *C. maximum*; (H) dead *C. maximum* with the shells embedded in the coral structure; (J) shell encrusted with epifauna and (K) an individual of *C. maximum* outside the shell

1.2.1.1.2 *Ceraesignum maximum*'s feeding strategy

C. maximum uses a mucus net as its feeding strategy, which can trap food particles (Morton, 1965; Hughes and Lewis, 1974; Kappner, 2000). The mucus threads are constantly spread over the substratum under the turbulent action of currents and waves, but do not tear apart, apparently quite resistant to the force of the water (Hughes and Lewis, 1974; Kappner, 2000). After the exposure of the net for some time, the net is withdrawn and consumed at intervals, while the production of the mucus filaments continues (Kappner, 2000). The process of retracting the net is conducted with the use of the jaws and the rotation of the head, while the ingestion is done by the radula (Hadfield et al., 1972; Hughes and Lewis, 1974). The oesophagus, which is long and elastic, permits the ingestion of the mucus with speed which is faster than its processing in the stomach (Kappner, 2000). The complete feeding cycle (web secretion, exposure, and retraction) takes around 35 minutes (Kappner, 2000). Usually this organism is active 24 hours per day (Hughes and Lewis, 1974; Kappner, 2000), but its activity decreases in the morning (Kappner, 2000), and in this period snail worms have been observe to withdraws inside the shell for a time of 1 to 15 minutes, and consequently the production of mucus is ceased (Hughes and Lewis, 1974; Kappner, 2000).

Furthermore, analyses conducted on the particles trapped on the mucus shown that the animal's diet consists principally of plankton, meiobenthos, and detritus (Kappner, 2000). Additionally, bioactive compounds have been detected in the mucus and antibiotic effects may decrease the deterioration of plankton by bacteria; however, these compounds' actions on coral tissue degradation are still unknown (Kloppel et al., 2013).

1.2.1.1.3 Reproduction and larvae of *Ceraesignum maximum*

A previous study has suggested a shift in the sex ratio of *C. maximum* from small sized individuals that are mostly male, to larger individuals, which are female, supporting a protandric hermaphroditism type of reproduction (Phillips and Shima, 2009). It should be noted that more analyses have to be conducted, especially histologically examinations of the gonads, to confirm this strategy (Phillips and Shima, 2009).

The female broods the larvae in capsules, that can range in number from 1 to 58 (Phillips and Shima, 2009), and which are attached to the internal wall of the tube (Hughes and Lewis, 1974). Moreover, this species commonly forms egg capsules at different stages of development at the same time (Phillips and Shima, 2009).

When the brooding last-stage capsule is reached, the eggs are wrapped in mesh-side cages and afterword the swimming larvae are released (Phillips and Shima, 2009). However, it has also been suggested in a previous study that this organism might release crawling juveniles (Hadfield et al., 1972), but further observations are needed to confirm this matter.

After hatching, larvae have a well-developed protoconch and velum and are able to feed immediately, and they can survive up to 10 days without food (Phillips and Shima, 2009), although nutriment it is required for the metamorphosis process (Phillips, 2011). Moreover, since the planktonic stage is short, this limits the dispersal of the larvae (Phillips and Shima, 2009).

In laboratory tests, larvae 10 days after hatching and after 1 day of exposure to coral rubble lost their vela and crawled on the substrata, and once they settled the process of metamorphoses took place, although the process of settlement in the field is poorly known (Phillips, 2011).

1.2.1.1.4 Negative effects caused by *Ceraesignum maximum* towards corals

As previously mentioned, the presence of *C. maximum* can pose as a threat to corals, especially when present in high densities (Shima et al., 2010, 2013). These high densities, also probably due to mucus nets, can cause a reduction of coral skeletal development by up to 81%, and to coral survival by up to 52%

(Shima et al., 2010). Moreover, these effects suggest that interactions between these two organisms can potentially cause a shift in the coral community (Shima et al. 2010), but further studies are needed on the matter (Shima et al. 2013).

Furthermore, anomalies of coral structure due to snail worms have been reported from the Red Sea (Zvuloni et al., 2008) and in Moorea (French Polynesia) (Shima et al.,2010, 2013, 2015).

Additionally, the mucus nets of different individuals can merge together to form a sheet that can became thick and dense to the point that it can trap gas bubbles, which may touch the coral surface. Various stages of degradation were visible from mild loss of pigmentation and flattening of structures, to more deleterious degradation such as the exposure of dead coral overgrown by algae (Kloppel et al., 2013).

Corals, however, are not completely undefended against *C. maximum*, and laboratory experiments conducted by Phillips et al. (2014) showed that larvae are unable to establish themselves on living coral and that different species of coral can be a source of larval mortality. Indeed, *C. maximum* settles on non-living substrata (Phillips, 2011; Phillips et al. 2014), and for this reason it can take advantage of small disturbances that may cause the death of coral to

establish itself on a habitat that that would be otherwise unavailable (Phillips et al. 2014).

As well, the guard crab *Trapezia serenei* has been shown to alleviate the effects of worm snails on corals (Stier et al., 2010).

1.3 Aim of the study

Several aspects of *Ceraesignum maximum* are still unknown and others need further observation, especially regarding the species' genetics. For example, regarding population genetic studies only one study has been conducted. This study demonstrated that *C. maximum* made a good model species to survey gene flow over short distances on coral reefs, in this case across Palau (Soliman et al., 2019).

The aim of the current study was to follow a similar approach to Soliman et al. (2019) in investigating the population genetic diversity on the coral reefs of Okinawa, in particular focusing on the differentiation between the west and east coasts. The two shores do not share the same characteristics, as the west coast in under the influence of the Kuroshio Current and has fringing coral reefs; while the east does not benefit from the Kuroshio Current, and as a result the water temperature is lower and there are several wide sloping bays (White et al., 2016).

Also, the current might play a role in connecting the different sites, as suggested for *Sticopus chloronotus* (greenfish holothurian), where absence on the east coast may have been the source of unique populations (Soliman et al., 2016a)

Moreover, Soliman et al. (2016b) in a study conducted on another holothurian species, *Holoturia edulis*, suggested the possibility that physical barriers and

biogeographical characteristics of Okinawa Island might cause a decrease in gene flow.

In addition, in the current study we also investigated the genetic diversity via comparison of anthropogenically impacted sites with more pristine ones. Okinawa Island, especially the southern part, over the past years has faced extensive costal development and land-filling activities, which have reduced and degraded habitats and altered the water quality, causing a decrease in marine biodiversity in the reefs closest to the shore (Reimer et al., 2015). Consequently, human impacts may be the cause of decreases in genetic diversity (Soliman et al., 2016b).

Chapter Two

MATERIALS AND METHODS

2.1 Sample collection

Different sites around Okinawa were investigated; however, the presence of *C. maximum* was confirmed only at seven locations along Okinawa coasts and neighboring islands (Table 1) (Fig. 4). A total of 85 specimens of *Ceraesignum maximum* were collected, by snorkeling and SCUBA diving, mostly from inner reefs at depth ranging between 0 and 5 m, from April 2018 to August 2018 (Table 1)

The organisms gathered were all relatively large (operculum: ~ 1.55 cm, Hughes and Lewis 1974), probably suggesting that they were female, but no measurements or histologically analyses were conducted to determine the dimension and the sex of the animals.

For the removing of the snail worms from living corals without damaging the coral colony, different tools were used such as hooks and a diving knife; and only in one occasion hammer and chisel for extraction from coral rock (Table 1).

Upon collection, the samples were immediately stored in 99.5% ethanol for subsequent genetic analyses, and sorted afterword in laboratory, where each specimen was assigned with a field collection code.

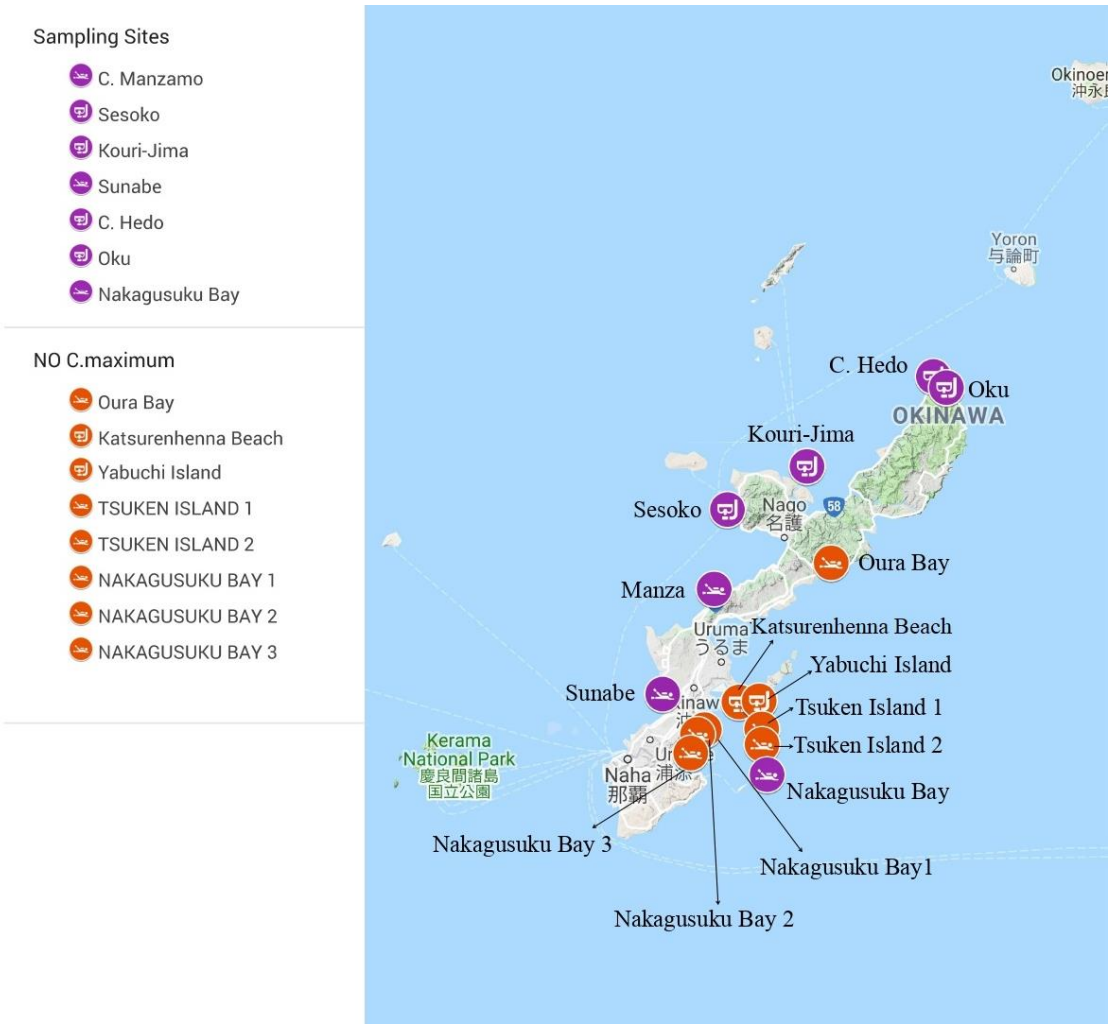

Figure 4. Okinawa sampling map. In purple are the location were *C. maximum* was found. In Red are the site investigated, but the snail worm was not found. The icons represent snorkeling or SCUBA diving activities; for more information see table 1

Table 1. Sampling information of specimens used in this study

LOCATION	LATITUDE	LONGITUDE	N° OF SPECIMENS	SUBSTRATE	HABITAT	DEPTH (Meters)	COLLECTION	COLLECTION TOOLS
1_CAPE MANZAMO	26°30'15.3 N	127°50'36.59" E	7	CORAL	INNER REEF	5	SCUBA DIVING	HOOK
2_SESOKO-JIMA	26°38'21.2" N	127°52'8.04" E	9	CORAL/CORAL ROCK	INNER/ PATCH REEF	0.5 - 1	SNORKELING	HOOK
3_KOURI-JIMA	26°42'47.3" N	128°1'14.35" E	9	CORAL	INNER REEF	1 – 2	SNORKELING	HOOK
4_SUNABE	26°19'27.3" N	127°44'43.61" E	11	CORAL ROCK	INNER REEF	0.8 - 1.8	SCUBA DIVING	HOOK
5_NAKAGUSUKU BAY	26°11'6.58" N	127°56'32.04" E	7	CORAL ROCK	CREST REEF	2	SCUBA DIVING	HAMMER AND CHISEL
6_CAPE HEDO	26°51'59.8" N	128°15'44.14" E	24	CORAL	INNER REEF	1 - 1.5	SNORKELING	DIVING KNIFE
7_OKU	26°50'49.1" N	128°17'14.54" E	18	CORAL	INNER REEF	2	SNORKELING	DIVING KNIFE

2.2 DNA extraction and PCR

DNA was extracted from the head tissue, because in most cases it was the only part of the organism available. For this procedure two extraction methods were used, the DNeasy Blood and Tissue Kit (Qiagen) in which the manufacturer's manual was followed with few adjustments, and an ammonium acetate protocol.

With the Qiagen kit a small fragment of tissue (1 to 2 mm^3) was cut with sterilized disposable scalpel and tweezers and placed in a 1.5 ml microcentrifuge tube. Then 180 µl of lysing buffer (Buffer ATL) was added with 20 µl proteinase K, vortexed and placed in the water bath or incubator at 56°C overnight until the tissue was completely digest. Next, 200 µl of another lysing buffer (Buffer AL: Containing guanidine hydrochloride) was added and afterwards 200 µl of ethanol (100%) was combined to the mix. Then the content inside the microcentrifuge tubes was taken out with the use of a pipette and transferred into a DNeasy Mini spin column placed in a 2 ml collection tube and centrifuged at ≥6000 x g (8000 rpm) for 3 min. The flow-through was discarded together with the collection tube. In the next passage, the spin columns were placed in a new 2 ml collection tubes and 500 µl of wash buffer (Buffer AW1 diluted before use with 125 ml of ethanol 100%) was added and the tubes were spun in the centrifuge for 1 min at ≥6000 x g and the collection

tubes and the flow-through were removed. Again new 2 ml collection tubes were placed under the spin columns and 500 µl of a second wash buffer (Buffer AW2 diluted before use with 160 ml of ethanol 100%) were pipetted and centrifuged for 4 minutes at 20,000 x g (14,000 rpm) and the collection tubes containing the flow-through were replaced with new 1.5 ml microcentrifuge tubes and the DNA was eluted by the addition of 100 µl Buffer AE and then incubated at room temperature (15–25°C) for 10 minutes and later centrifuged for 1 min at ≥6000 x g (Fig. 5). The extracted DNA was stored in freezer at -20°C.

Figure 5. Process of how to extract the DNA with the DNeasy Blood and Tissue Kit (Qiagen)

The second extraction method with ammonium acetate consisted of cutting a small tissue part (1-2 mm^3) and added 300 µl of lysis solution (pH 8, 10 mM Tris base, 100 mM EDTA, 2% SDS, 1 liter of milliQ water), then 5 µl of Proteinase K was added and the samples incubated overnight at 55°C. Afterward, 300 µl of ammonium acetate were pipetted into the tubes and let to rest in ice or freezer for 30 minutes. Then the samples were centrifuged at 14,000 rpm for 10 minutes and the supernatant transferred to new chilled 1.5 ml tubes, where 600 µl of absolute isopropanol was added and gently inverted. Another centrifugation was required at 13,000 rpm for 10 minutes and the supernatant was removed and to the remaining pellet 600 µl of chilled ethanol 70% were added. Moreover, the samples were placed in a centrifuge for 10 minutes at 13,000 rpm and afterward the ethanol was removed and the samples were dried in a vacuum for 1 hour or more. After, the DNA was resuspended with 100 µl with Buffer AE and stored in the refrigerator.

As DNA marker, the cytochrome C oxidase subunit I of mitochondrial DNA (COI mtDNA) was utilized, which was amplified by polymerase chain reaction (PCR) using the degenerate primers LCO1490 (5'-DKT CDA CWA AYC ATA ARG AYA TTG G-3') and HC02198 (5'- TAW ACY TCH GGR TGH CCR AAR AAY CA-3') (Folmer et al., 1994). PCRs were done in a 20 µl volume,

containing approximately 4.8 µl of distilled water, 10 µl of Master Mix (consisting of Taq DNA Polymerase, PCR Buffer and dNTPs) (Qiagen), 1 µl of each primer, 1 µl of Coral Red Buffer dye, 1.2 µl of $MgCl_2$ and 1 µl of DNA template from the samples. Amplification protocol followed was: an initial denaturation at 10 min at 95°C, follow by 40 cycles of 95°C for 45 s, 40°C for 45 s and 72°C for 90 s with a final extension at 72°C for 10 min (Folmer et al., 1994), however some of the parameters where changed such as the increase of cycles from 35 to 40; the raising of annealing temperature up to 48°C and is some cases up to 50°C, and in the first part of the extension the time was increased up to 1 minute and 30 seconds. Amplification success was verified by electrophoresis runs on 1.5 % agarose gel and the PCR products that had positive results were then purified with an ExoSAP PCR clean-up protocol for 20 minutes at 37 °C, followed by 30 minutes at 83°C. After this process the cleaned samples were sequenced both in forward and reverse directions by Fasmac (Kanagawa, Japan).

2.3 Data analyses

The sequences obtained were trimmed with MEGA version 7.0 (Kumar et al., 2016) and subsequently compared with the sequences of *C. maximum* already

recorded in the databank of NCBI (National Center for Biotechnology Information) with the use of nBlast (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The sequences were checked to make sure no stop-codons were present with the software Geneious version 11.1.5 (Kearse et al. 2012). Afterwords, sequences were aligned using MUSCLE through MEGA 7.0 (Kumar et al., 2016) and with the same program a neighbor-joining phylogenetic tree was created. The number of haplotypes (H), the number of segregation sites (S), and the haplotype diversity (Hd) were detected from the sequences with the use of DnaSP version 6 (Rozas et al. 2017).

The haplotypes were then used in PopART (Population Analysis with Reticulate Trees) version 1.7 (Leigh and Bryant, 2015), which is a population genetics software package, to create a median-joining haplotype network.

The seven populations were then divided in various groups and tested for the differentiations between them (among groups, among populations within groups and within population), with the application of an analysis of molecular variance (AMOVA) run in Arlequin version 3.5.2.2 (Excoffier and Lischer, 2010). AMOVA is a statistical model which produces estimations of variance components and F-statistic analogs and it is used to detect population differentiation (Excoffier et al., 1992)

Chapter Three

RESULTS

The PCR products that had positive results on the electrophoresis gel and thus sent to sequencing numbered 61, and of these only two were obtained with the ammonium acetate DNA extraction method. The numbers of sequences obtained were; 7 for Cape Manzamo (hereafter Manza), 8 for Sesoko, 9 for Kouri, 11 for Sunabe, 4 for Nakagusuku, 13 for Cape Hedo, and 9 for Oku.

Only the forward sequences (537 bp long) were taken in consideration for the subsequent analyses, due to the bad quality of the reverse, perhaps due to the degenerate nature of the primer, preventing us from assembling them with the forward sequence.

The neighbor-joining phylogenetic tree (Fig. 6), with the sequence of *C. maximum* (NC_014583) (Rawlings et al., 2010) previously recorded in NCBI as outgroup, shows well defined clades for some sampling areas such as Manza, Kouri and Sunabe. However, mixed clades were also evident for sequences from specimens from Sesoko, Sunabe, Nakagusuku Bay, Cape Hedo and Oku (Fig. 6).

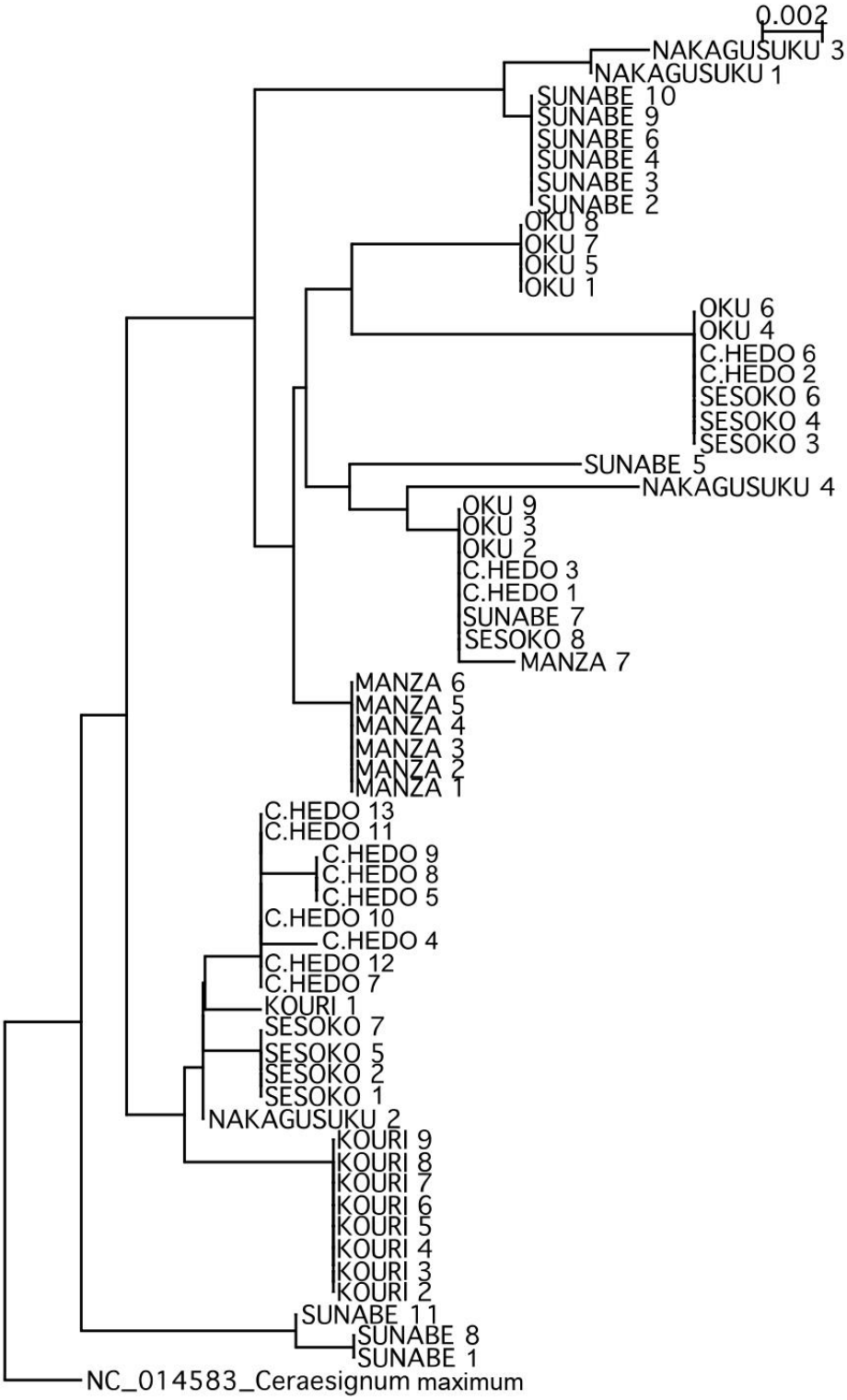

Figure 6. The neighbor-joining phylogenetic tree of COI mitochondrial DNA sequences, with the sequence of *C. maximum* (NC_014583) from NCBI as outgroup

Across the populations of *C. maximum* analyzed, a total of 19 haplotypes (H) (Fig. 7) and 44 segregation sites (S) were found. The division of the haplotypes was as follows: Manza H01 and H02; Sesoko H03, H04, H05; Kouri H06, H07; Sunabe H05, H08, H09, H10, H11; Nakagusuku H12, H13, H14, H15; C. Hedo H04, H05, H16, H17, H18; and Oku H04, H05 and H19 (Table 2).

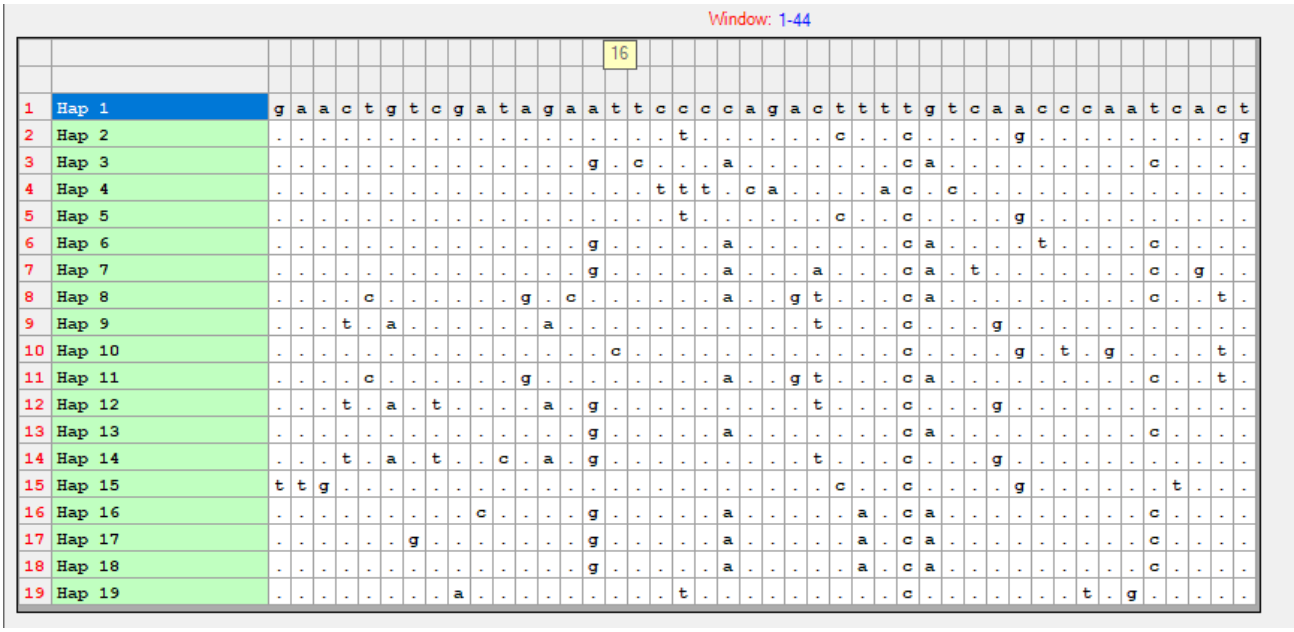

Figure 7. Haplotype found with the program DnaSP

The highest numbers of haplotypes were observed at Sunabe and C. Hedo (H = 5), while the lowest were from Manza and Kouri (H = 2), while Sesoko and Oku had 3 H each and Kouri and Nakagusuku had respectively 4 H.

The haplotype diversity (Hd) was highest in Nakagusuku (Hd = 1), while similar values were observed in Sesoko (Hd = 0.67857), in Sunabe (Hd =

0.70909), in C. Hedo (Hd = 0.80769) and in Oku (Hd = 0.72222), while the lowest were in Manza (Hd = 0.28571) and in Kouri (Hd = 0.22222) (Table 2).

Table 2. Genetic diversity indices of 61 mtDNA sequences (537 bp long) of speciemens of C. maximum from 7 sampling sites across Okinawa

LOCATION	N° OF SEQUENCES	NUMBER OF HAPLOTYPES (H)	NUMBER OF SEGREGATION SITES (S)	HAPLOTYPE DIVERSITY (Hd)
C. MANZAMO	7	2	5	0.28571
SESOKO	8	3	14	0.67857
KOURI-JIMA	9	2	4	0.2222
SUNABE	11	5	19	0.70909
NAKAGUSUKU BAY	4	4	17	1.00
C. HEDO	13	5	16	0.80769
OKU	9	3	11	0.72222
OVERALL	61	19	44	0.93115

The median-joining haplotype network (Fig. 8), obtained in PopART (Leigh and Bryant, 2015), of 19 haplotypes found among the seven populations showed that the shared haplotypes were H04, found in Sesoko, C. Hedo and Oku; and H05, which was at Sesoko, Sunabe, C. Hedo and Oku.

In the network analyses figure, the dimensions of the circles represent the numbers of samples collected that had the same haplotype, and thus bigger

circles have more sequences, while the smaller circles are single individuals (Fig. 8).

In consideration of this, in Manza H01 was observed in six sequences, and H02 in only one sequence. In Sesoko H03 was found in in four samples, while H04 was present in three organisms and H05 in one. Kouri had H06 and H07, respectively, divided into one and eight sequences. In Sunabe we assessed that H05, H10 and H11 were in one sample each and H08 in two sequences, while H09 was present in six.

Nakagusuku Bay had four individuals examined and each one of them had a different haplotype (H12, H13, H14, H15). C. Hedo had H04 and H05 which were respectively two sequences each, H16 that was present only in one sample, H17 was in three organisms, while H18 was in five sequences. Oku had a total of nine samples that were divided in two for H04, three for H05 and four for H19 (Fig 8).

Moreover, the smaller perpendicular bars on the branches show the pair base differences between one haplotype to the other (Fig.8). Some of the haplotypes differed only by a pair base from one another (e.g. H03 and H06 from H13 or H02 from H05), while other haplotypes differed by a higher number of mutations, for example H05 had five different base pairs from H15 (Fig 8).

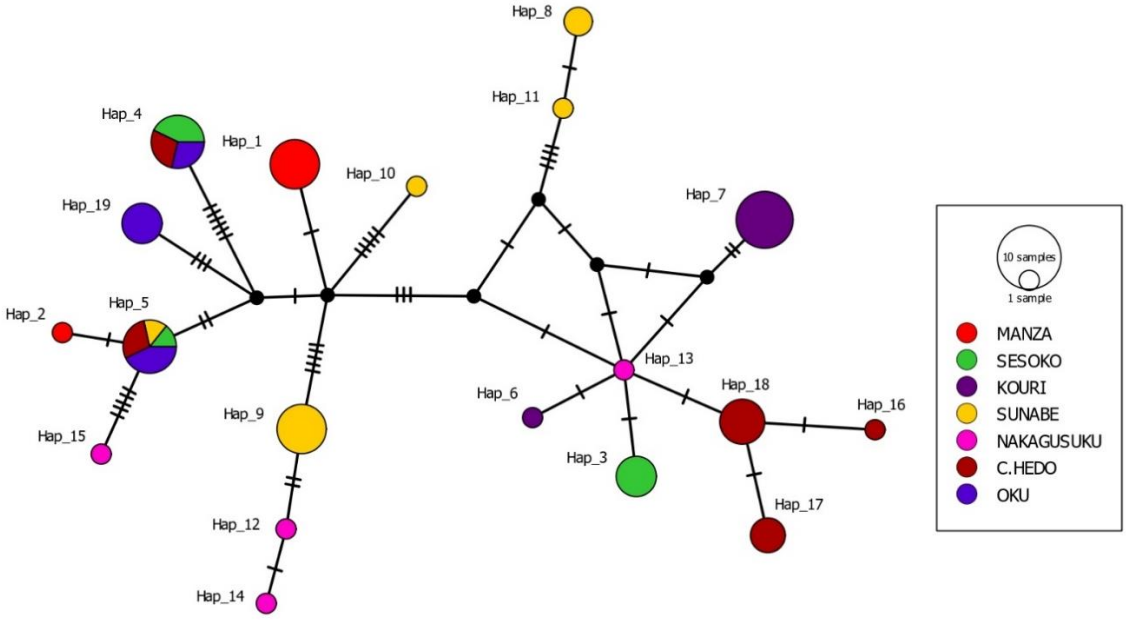

Figure 8. Median-joining Haplotype network constructed with COI mitochondrial DNA sequences of 61 *Ceraesignum maximum* individuals among 7 populations

The AMOVA was performed with the division of the seven populations in reflection of their location on the west and east coasts of Okinawa Island. Accordingly, sampling sites on the west were Manza, Sesoko, Kouri and Sunabe, while on the east they were Nakagusuku Bay, C. Hedo and Oku. An additional AMOVA was performed where C. Hedo and Oku were moved to the west coast, given their position at the northern end of Okinawa Island.

Another AMOVA evaluation was made to test the differences between areas anthropogenically impacted (Nakagusuku Bay and Sunabe) and the sites that have maintained their pristine conditions (Manza, Sesoko, Kouri, C. Hedo and Oku).

Afterword, the same AMOVA analyses were redone but the samples from C. Hedo and Oku were combined and considered as one population, since their locations are only 2 km apart.

The results obtained were as follows:

COMPARISON BETWEEN WEST AND EAST COASTS (west coast: Manza, Sesoko, Kouri, Sunabe; east coast: Nakagusuku Bay, C. Hedo and Oku)

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation
Among groups	1	0.375	-0.00553 Va	-1.15
Among populations within groups	5	2.250	-0.01531 Vb	-3.19
Within populations	17	8.500	0.50000 Vc	104.35
Total	23	11.125	0.47917	

Fixation Indices

FSC : -0.03158
FST : -0.04348
FCT : -0.01154

Significance tests (1023 permutations)

Vc and FST : P(rand. value < obs. value) = 0.74585
P(rand. value = obs. value) = 0.25415
P-value = 1.00000+-0.00000

Vb and FSC : P(rand. value > obs. value) = 0.59531
P(rand. value = obs. value) = 0.40469
P-value = 1.00000+-0.00000

Va and FCT : P(rand. value > obs. value) = 0.67253
P(rand. value = obs. value) = 0.17302
P-value = 0.84555+-0.00987

COMPARISON BETWEEN WEST AND EAST COASTS, in which C. Hedo and Oku on the east coast (west coast: Manza, Sesoko, Kouri, Sunabe, C. Hedo and Oku; east coast: Nakagusuku Bay)

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation
Among groups	1	0.575	0.02778 Va	5.56
Among populations within groups	5	2.050	-0.02778 Vb	-5.56
Within populations	17	8.500	0.50000 Vc	100.00
Total	23	11.125	0.50000	

Fixation Indices

FSC : -0.05882
FST : -0.00000
FCT : 0.05556

Significance tests (1023 permutations)

Vc and FST : P(rand. value < obs. value) = 0.72141
P(rand. value = obs. value) = 0.27859
P-value = 1.00000+-0.00000

Vb and FSC : P(rand. value > obs. value) = 0.79765
P(rand. value = obs. value) = 0.20235
P-value = 1.00000+-0.00000

Va and FCT : P(rand. value > obs. value) = 0.00000
P(rand. value = obs. value) = 0.14272
P-value = 0.14272+-0.01035

COMPARISON BETWEEN NON-IMPACTED AND IMPACTED AREAS (non-impacted area: Manza, Sesoko, Kouri, C. hedo and Oku; impacted area: Sunabe and Nakagusuku Bay)

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation
Among groups	1	0.525	0.01136 Va	2.33
Among populations within groups	5	2.100	-0.02493 Vb	-5.13
Within populations	17	8.500	0.50000 Vc	102.79
Total	23	11.125	0.48643	

Fixation Indices

FSC : -0.05248
FST : -0.02790
FCT : 0.02335

Significance tests (1023 permutations)

Vc and FST : P(rand. value < obs. value) = 0.76442
P(rand. value = obs. value) = 0.23558
P-value = 1.00000+-0.00000

Vb and FSC : P(rand. value > obs. value) = 0.72630
P(rand. value = obs. value) = 0.27370
P-value = 1.00000+-0.00000

Va and FCT : P(rand. value > obs. value) = 0.13881
P(rand. value = obs. value) = 0.03324
P-value = 0.17204+-0.01204

COMPARISON BETWEEN WEST AND EAST COASTS (C. Hedo and Oku considered as one population) (west coast: Manza, Sesoko, Kouri, Sunabe; east coast: Nakagusuku Bay, C. Hedo-Oku)

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation
Among groups	1	0.402	-0.00648 Va	-1.33
Among populations within groups	4	1.917	-0.00627 Vb	-1.29
Within populations	16	8.000	0.50000 Vc	102.62
Total	21	10.318	0.48725	

Fixation Indices

FSC : -0.01269
FST : -0.02617
FCT : -0.01331

Significance tests (1023 permutations)

Vc and FST : P(rand. value < obs. value) = 0.51613
P(rand. value = obs. value) = 0.48387
P-value = 1.00000+-0.00000

Vb and FSC : P(rand. value > obs. value) = 0.23363
P(rand. value = obs. value) = 0.76637
P-value = 1.00000+-0.00000

Va and FCT : P(rand. value > obs. value) = 0.58944
P(rand. value = obs. value) = 0.05572
P-value = 0.64516+-0.01455

COMPARISON BETWEEN WEST AND EAST COASTS, C. Hedo and Oku considered as one population and belonging to the west coast (west coast: Manza, Sesoko, Kouri, Sunabe, C. Hedo-Oku; east coast: Nakagusuku Bay)

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation
Among groups	1	0.540	0.01626 Va	3.25
Among populations within groups	4	1.778	-0.01626 Vb	-3.25
Within populations	16	8.000	0.50000 Vc	100.00
Total	21	10.318	0.50000	

Fixation Indices

FSC : -0.03361
FST : -0.00000
FCT : 0.03252

Significance tests (1023 permutations)

Vc and FST : P(rand. value < obs. value) = 0.49267
P(rand. value = obs. value) = 0.50733
P-value = 1.00000+-0.00000

Vb and FSC : P(rand. value > obs. value) = 0.60802
P(rand. value = obs. value) = 0.39198
P-value = 1.00000+-0.00000

Va and FCT : P(rand. value > obs. value) = 0.00000
P(rand. value = obs. value) = 0.14467
P-value = 0.14467+-0.01309

COMPARISON BETWEEN NON-IMPACTED AND IMPACTED AREAS (C. Hedo and Oku considered as one population) (non-impacted area: Manza, Sesoko, Kouri, C. Hedo-Oku; impacted area: Sunabe and Nakagusuku Bay)

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation
Among groups	1	0.472	0.00209 Va	0.43
Among populations within groups	4	1.846	-0.01151 Vb	-2.35
Within populations	16	8.000	0.50000 Vc	101.92
Total	21	10.318	0.49058	

Fixation Indices

FSC : -0.02356
FST : -0.01920
FCT : 0.00426

Significance tests (1023 permutations)

Vc and FST : P(rand. value < obs. value) = 0.52493
P(rand. value = obs. value) = 0.47507
P-value = 1.00000+-0.00000

Vb and FSC : P(rand. value > obs. value) = 0.45552
P(rand. value = obs. value) = 0.54448
P-value = 1.00000+-0.00000

Va and FCT : P(rand. value > obs. value) = 0.28543
P(rand. value = obs. value) = 0.05963
P-value = 0.34506+-0.01905

Looking at the AMOVA results, all show that the highest variation was registered within the populations, and there was not significant differences between the locations on both coasts, and that there were no differences even when C. Hedo and Oku were considered as one population. Moreover, a similar AMOVA outcome was observed when the sites were compared as polluted and natural sites, with no significant differences observed.

Chapter Four

DISCUSSION AND CONCLUSIONS

Our investigation of population genetics regarding the vermetid gastropod *Ceraesignum maximum* showed interesting results, different from those previously observed from the reefs of Palau. We assessed that the seven populations of *Ceraesignum maximum* along Okinawa coasts probably represent one mixed population. Our AMOVA results confirm that apparently there were no differences in the seven sites, concerning both the positions on the east or west coasts, or with regards to the anthropogenically impacted areas and more natural sites.

Moreover, the haplotype network suggests that the population in Okinawa was not recently established, and is stable, due to the presence of unique haplotypes sites and it also seems that there are not any recent expansion patterns (Fig. 8). Since the absence of previous work concerning the same species in Okinawa, we compared our results with other similar research conducted on different species of invertebrates, to evaluate if we have a similar outcome.

If we take in consideration the study conducted by Soliman et al. (2016b) on *Holoturia edulis* on Okinawa, this previous work shows a moderate number of haplotypes shared across the sites; however, the shifts in their frequencies still showed a genetic isolation among the sites. Moreover, the results of the research made by Soliman et al. (2016a) on *Stichopus chloronotus,* in which the markers utilized were the 16S ribosomal DNA (three haplotypes) and nuclear histone H3 gene (one haplotype), revealed a low genetic diversity in this species of sea cucumber. Furthermore, haplotype frequency differences between Okinawan shores was detected, and the source of this was speculated to be due to habitat characteristics or oceanographic barriers (Soliman et al., 2016a).

The low genetic diversity observed could also have been caused in part by the anthropogenic impacts at some sites along Okinawa coasts (Soliman et al., 2016a).

Similar outcomes of genetic differentiation between Okinawa's west and east coasts have been observed in the holothurian *H. edulis* (Soliman et al., 2016b) and in the amphipod *Leucothoe vulgaris* (White et al., 2016).

In our haplotype network it is possible to see that there are only two shared haplotypes (H04, H05), while the single haplotypes unique to each site are the majority, underlining an interesting trend with many unique alleles (Fig.8). We

speculate that ours results would have shown significance in AMOVA analyses with a higher number of specimens.

Overall, the results here are quite different from those recently observed in Palau, where a separation between inner and outer reef populations was confirmed (Soliman et al., 2019), while in our case no significant differences among the sites was detected. However, it has to be verified if a similar division can be made for Okinawa, since the fringing reefs are not as well developed as in Palau. As well, it should be taken in consideration that Okinawa is located in a location with subtropical monsoons and typhoons in the summer. How this would affect connectivity of *C. maximum* compared to results from tropical Palau remains to be assessed.

However, the lack of statistical significance in our results due to a relatively low number of samples could also be a reason why we did not observe any differentiation in populations, particularly given the fact that we observed many unique haplotypes at some sites, and given the evident stability of the populations. It may be that with an increase of specimens, significance levels may change dramatically, and we may observe genetic differentiation across different sites.

In conclusion, our study can be considered as a preliminary investigation on the population genetics of *Ceraesignum maximum* in Okinawa. Our data, even

if it cannot be considered sufficient to determinate a clear image of the genetic flow on the coral reefs around the island, has shown interesting patterns regarding *C. maximum* populations that warrant more investigation. For this reason, it essential to collect more specimens to increase the numbers to create a more solid background when conducting analyses. Specifically, it necessary to locate more sites where there are *C. maximum* on the east coast, as in our research one weak point when running the AMOVA was the lack of samples coming from the east coast.

Moreover, more statistical analyses with such future data, such as the estimation of Fu's F, and principle component analyses (PCA), would be required to more comprehensively assess the results obtained.

Finally, for *C. maximum*, it is also important to evaluate other aspects such as distribution, ecology, and morphology (e.g. Hughes and Lewis, 1974; Kappner, 2000; Phillips and Shima, 2009; Shima et al. 2010, 2013; Golding et al., 2014), since multiple aspects of this organisms are still relatively unknown in general and in particular for the coral reefs surrounding Okinawa.

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