CONSTRAINING THE PHYSIOLOGICAL, GENETIC, AND SYMBIOTIC ADAPTATION OF AN INVASIVE FORAMINIFERA IN THE MEDITERRANEAN SEA

Débora Silva Raposo

DISSERTATION

To obtain the academic degree of Doctor of Natural Sciences

-Dr. rer. nat.-

Department of Geosciences (FB5) at the University of Bremen

December 2022

Reviewers:

Prof. Dr. Michal Kucera (*MARUM, University of Bremen, Bremen*)

Prof. Dr. Micah Dunthorn (*Natural History Museum, University of Oslo, Oslo*)

Date of colloquium: February 27, 2023

marum
Zentrum für Marine Umweltwissenschaften

University of Bremen

Affirmation in lieu of an Oath

according to § 5 (5) of the Doctoral Degree Rules and Regulations of 18 June, 2018

I, Débora Silva Raposo (Address [redacted]), Student ID. [redacted]

With my signature, I affirm in lieu of an oath that I prepared the submitted dissertation independently and without illicit assistance from third parties, that I appropriately referenced any text or content from other sources, that I used only literature and resources listed in the dissertation, and that the electronic (PDF) and printed versions of the dissertation are identical.

I affirm in lieu of an oath that the information provided herein to the best of my knowledge is true and complete.

I am aware that a false affidavit is a criminal offence which is punishable by law in accordance with § 156 of the German Criminal Code (StGB) with up to three years imprisonment or a fine in case of intention, or in accordance with § 161 (1) of the German Criminal Code with up to one year imprisonment or a fine in case of negligence.

_____ _____

Place, Date Signature

Every living thing is a masterpiece, written by nature and edited by evolution.

Neil deGrasse Tyson

American astrophysicist, author, and science communicator

Content

Acknowledgments

I would like to express my deepest gratitude to

My dear supervisor Prof. Dr. Michal Kucera for believing in me and investing in this project, for leading me on my scientific journey with his mentoring, and for the fantastic writing sessions.

My dear co-supervisor Dr. Raphaël Morard for his unlimited support and patience to guide me in my project, for his valuable advice on my academic career, for thoroughly teaching me the genetic lab methods, for his friendship and the good laughs in the office, and of course for driving me around the Sicilian and Israeli coasts to get my samples.

My co-authors on the different manuscripts, without them this mission would have simply been impossible. I would especially like to recognize Dr. Christiane Schmidt who taught me and gave major advice on the physiological experiment's methods; Dr. Christiane Hassenrück for all her assistance and patience to teach me the bioinformatics methods, but more than that, for the good moments we spent together during this time; and Dr. Danna Titelboim, for instigating my creativity and brainstorming together, for her friendship, and for all her support during the field trip in Israel.

My working group AG Kucera for the valuable scientific discussions that enabled me to grow in my academic life and for the nice lunch and coffee breaks.

My student-fellows Anne, Dakota, Eldo, Elinor, Franziska, Mattia, Nurshan, Peter, Qingxia, and Tamara, who were present at different times of my PhD but were all important to me by helping with paperwork, sharing a good talk, long days in the lab, and even bouldering sessions.

My friends in Bremen Brad, Carol, Débora, Guilherme, Gustavo, Jéssica, Pauline, and Sandy, who were a big part of my resilience to this pandemic/immigration journey in the last 3.5 years by sharing special moments that I will always keep in my heart.

My dear parents, Kátia and Roberto, who always supported me in the best way possible, and even with an ocean now separating us, never ceased to be present in my life.

My love and husband Igor for dreaming of this moment together with me, for making my days lighter with his company, for understanding the absent moments resultant of the academic career, and for always knowing what to say in the toughest times of this journey.

Summary

Global change may directly or indirectly influence biological invasions by increasing the chances of the introduction and establishment of alien species into new habitats. Such processes pose major threats to biodiversity and the resulting changes in ecosystems may have serious functional and economic impacts. A dramatic migration of tropical species from the Red Sea into the Mediterranean Sea was triggered when the Suez Canal was opened in 1869. Some invaders became highly abundant in their new locations, such as the benthic foraminifera *Amphistegina lobifera*. In its newly conquered Mediterranean habitat, the invasive populations are exposed to particularly low temperatures in winter (~13°C) and warmer temperatures in summer (~31°C), which represents a seasonality that exceeds the range of their native habitat (22°C in summer and 28°C in winter). This prolific calcifier that relies on photosymbiosis with diatoms has recently expanded its invasion front to Sicily and in a short period after its first appearance in 2016, the species became particularly abundant, sometimes reaching 50% of the benthic foraminifera assemblages. Therefore, to forecast future invasions and understand their impact in the new location it's crucial to comprehend the mechanisms by which a species might become a successful invader. In this context, this thesis aims to understand if the adaptation necessary to survive in the Mediterranean were already present in the foraminifera host and symbionts from the source population in the Red Sea (niche conservatism concept) or if the invasion success was due to rapid emergence of new adaptations in the invaders (inducing ecological niche shifts). For this, several approaches are applied, including genetic population structure, metabarcoding analyses, and physiological experiments.

To constrain if the invasion either induces or is facilitated by genetic adaptation of the foraminifera host or if the source population sustains key pre-adaptations to the new conditions, the population structure of *A. lobifera* across its invasive range was investigated (**Chapter 1**). The analyses revealed that the invading populations do not exhibit genetic divergence from the source population, and the invasion success in the Mediterranean Sea is associated with the combination of preadaptation, high dispersal ability, and ongoing reseeding from the Red Sea. However, given an increased genetic variation among individuals and decreased intragenomic variability, the invading populations appear to be affected by the invasion. The invasion, therefore, appears to be associated with a sustained change in reproductive strategy toward the abandonment of sexual reproduction, or with an increased failure in sexual reproduction and enhanced asexual reproduction, which could represent a long-term loss of adaptive potential.

In order to resolve if the local adaptation was enhanced by beneficial microbiome associations that the foraminifera acquired during the invasion, or if they carry the same associations as the source population, metabarcoding analyses of the foraminiferal microbiome (eukaryotic and bacterial) and the surrounding environmental DNA were applied (**Chapter 2**). Different bacterial microbiomes and diatom sequence variants were associated with the different host populations. However, the vast majority of the diatom sequence variants were absent in the environment. This implies that the foraminifera either preserve an ancestral stock of symbionts, with new strains emerging inside the host over the invasive range, or that they acquired their symbionts along the invasive range or during different seasons. These findings show that the algal symbiosis composition is flexible, and its modification may enable the holobiont to deal with the unique temperature regime of the invaded habitat.

Finally, to assess if the sustained exposure to colder winters in the invasive locations induced physiological adaptations in the foraminiferal holobiont, or if the success is a matter of a pre-adaptation inherited from the source population, a physiological experiment was performed (**Chapter 3**). In a four-week experiment, the physiological responses of the foraminifera host and its symbionts to colder temperatures (10, 13, 16, 19 °C + control at 25°C) were evaluated for the source, pioneer invaders, and invasion-front populations. The 13°C treatment represents the minimum temperature experienced in the invaded habitat and 10°C is an out-of-range treatment to assess whether the population has a higher adaptive range than already displayed. It was observed that the foraminifera host and its symbionts respond differently to cold stress. While there were no noticeable differences in survival and performance of the host (all populations showed low tolerance to cold temperatures < 16°C), the symbionts showed enhanced tolerance to cold temperatures (~13°C). In addition, the invasion front population symbionts were significantly resilient to even lower temperatures (10°C). Thus, the symbionts are likely the main responsible for the success of invasion under cold stress.

These results advance our understanding of the mechanisms regulating the invasion of a prolific calcifier benthic foraminifera in the Mediterranean Sea. The overall results indicate that the cold-tolerant photosymbiosis or the flexibility to form symbiosis with differently adapted diatoms is likely a key to the success of past and future migrations of this species. This can be used to refine models of ecological niche shifts during invasions and improve predictions of future marine invasions.

Zusammenfassung

Der globale Wandel kann sich direkt oder indirekt auf biologische Invasionen auswirken, indem er die Chancen für die Einführung und Etablierung gebietsfremder Arten in neuen Lebensräumen erhöht. Solche Prozesse stellen eine große Bedrohung für die biologische Vielfalt dar, und die daraus resultierenden Veränderungen in den Ökosystemen können schwerwiegende funktionelle und wirtschaftliche Auswirkungen haben. Die Eröffnung des Suezkanals im Jahr 1869 löste eine dramatische Migration tropischer Arten vom Roten Meer ins Mittelmeer aus. Einige der eingewanderten Arten, wie die benthische Foraminifere *Amphistegina lobifera*, kommen in ihren neuen Lebensräumen sehr zahlreich vor. In ihrem neu eroberten mediterranen Lebensraum sind die invasiven Populationen besonders niedrigen Temperaturen im Winter (~13 °C) und wärmeren Temperaturen im Sommer (~31 °C) ausgesetzt, was einer Saisonalität entspricht, die den Bereich ihres ursprünglichen Lebensraums (22 °C im Sommer und 28 °C im Winter) übersteigt. Diese produktiven Kalkbildner, die auf Photosymbiose mit Kieselalgen angewiesen sind, haben vor kurzem ihre Invasionsfront bis nach Sizilien ausgedehnt. Seit dem ersten Auftreten von *A. lobifera* im Jahr 2016 hat sich die Häufigkeit der Art deutlich erhöht und macht mitunter bis zu 50 % der benthischen Foraminiferen-Gemeinschaft aus. Um die künftige Ausbreitung invasiver Arten besser vorherzusagen und ihre Auswirkungen an der neuen Lokation zu verstehen, ist es entscheidend, die Mechanismen zu verstehen, die eine Art zu einem erfolgreichen Invasor macht. Das Ziel dieser Arbeit ist es daher zu verstehen, ob die für das Überleben im Mittelmeer notwendigen Anpassungen bereits in den Foraminiferen-Wirten und -Symbionten der Ausgangspopulation im Roten Meer vorhanden waren (Konzept des Nischenkonservatismus) oder ob der Invasionserfolg auf das schnelle Auftreten neuer Anpassungen in den Invasoren zurückzuführen ist (ökologische Nischenverschiebungen). Zu diesem Zweck werden verschiedene Methoden angewandt, darunter die genetische Populationsstruktur, Metabarcoding-Analysen und physiologische Experimente.

Um einzugrenzen, ob die Invasion entweder eine genetische Anpassung des Foraminiferen-Wirts hervorgerufen hat oder durch diese erleichtert wurde oder ob die Ausgangspopulation bereits wichtige Voranpassungen an die neuen Bedingungen enthält, wurde die Populationsstruktur von *A. lobifera* in ihrem gesamten Invasionsgebiet untersucht (**Kapitel 1**). Die Analysen ergaben, dass die eindringenden Populationen keine genetische Divergenz zur Ausgangspopulation aufweisen und der Erfolg der Invasion im Mittelmeer mit einer

Kombination aus Voranpassung, hoher Ausbreitungsfähigkeit und ständiger Wiederaussaat aus dem Roten Meer zusammenhängt. Da jedoch die genetische Variation zwischen den Individuen zunimmt und die intragenomische Variabilität abnimmt, ist davon auszugehen, dass die Invasion selbst die eindringenden Populationen beeinflusst. Die Invasion scheint also mit einer nachhaltigen Änderung der Fortpflanzungsstrategie verbunden zu sein, die zur Aufgabe oder dem zunhemenden Versagen der sexuellen Fortpflanzung und zu einer verstärkten asexuellen Fortpflanzung führt. Diese Änderung der Fortpflanzungsstrategie könnte einen langfristigen Verlust des Anpassungspotenzials zur Folge haben.

Um zu klären, ob die lokale Anpassung durch vorteilhafte Mikrobiom-Assoziationen verstärkt wurde, die die Foraminiferen während der Invasion erworben haben, oder ob sie die gleichen Assoziationen wie die Ausgangspopulation tragen, wurden Metabarcoding-Analysen des Mikrobioms der Foraminiferen (eukaryotisch und bakteriell) und der umgebenden Umwelt-DNA durchgeführt (**Kapitel 2**). Es wurden unterschiedliche bakterielle Mikrobiome und Diatomeen-Sequenzvarianten mit den verschiedenen Wirtspopulationen in Verbindung gebracht. Die überwiegende Mehrheit der Diatomeen-Sequenzvarianten war jedoch in der Umwelt nicht vorhanden. Dies deutet darauf hin, dass die Foraminiferen entweder einen Urbestand an Symbionten bewahren und neue Stämme im Laufe der Invasion im Wirt auftauchen, oder dass sie ihre Symbionten entlang der Invasionsstrecke oder während verschiedener Jahreszeiten erworben haben. Diese Ergebnisse zeigen, dass die Zusammensetzung der Algensymbionten flexibel ist und deren Veränderung den Holobionten in die Lage versetzen könnte, mit dem einzigartigen Temperaturregime des neuen Lebensraums zurechtzukommen.

Des Weiteren wurde ein physiologisches Experiment durchgeführt, um festzustellen, ob die anhaltende Exposition gegenüber kälteren Wintern an den eingedrungenen Standorten physiologische Anpassungen in den Holobionten der Foraminiferen hervorgerufen hat oder ob der Erfolg auf eine von der Ursprungspopulation vererbte Voranpassung zurückzuführen ist (**Kapitel 3**). In einem vierwöchigen Experiment wurden die physiologischen Reaktionen des Foraminiferenwirts und seiner Symbionten auf kältere Temperaturen (10, 13, 16, 19 °C + Kontrolle bei 25 °C) für die Ausgangspopulation, die Pionierinvasoren und die Populationen an der Invasionsfront untersucht. Die 13 °C-Behandlung entspricht der niedrigsten Temperatur, die in dem eingedrungenen Lebensraum herrscht, und die 10 °C-Behandlung ist eine Behandlung außerhalb des vorhandenen Temperaturbereichs, um festzustellen, ob die Population einen größeren Anpassungsbereich hat, als bereits angezeigt. Es wurde festgestellt,

dass der Foraminiferenwirt und seine Symbionten unterschiedlich auf Kältestress reagieren. Während es keine nennenswerten Unterschiede im Überleben und in der Leistung des Wirts gab (alle Populationen zeigten eine geringe Toleranz gegenüber kalten Temperaturen < 16°C), zeigten die Symbionten eine erhöhte Toleranz gegenüber kalten Temperaturen (~13°C). Darüber hinaus waren die Symbionten der Population an der Invasionsfront sogar bei niedrigeren Temperaturen (10°C) deutlich widerstandsfähiger. Somit sind die Symbionten wahrscheinlich die Hauptverantwortlichen für den Erfolg der Invasion bei Kältestress.

Diese Ergebnisse tragen zu einem besseren Verständnis der Mechanismen bei, die die Invasion dieser kalzifizierenden benthischen Foraminifere im Mittelmeer regulieren. Die Gesamtergebnisse deuten darauf hin, dass die kältetolerante Photosymbiose oder die Flexibilität, Symbiosen mit unterschiedlich angepassten Kieselalgen einzugehen, wahrscheinlich ein Schlüssel zum Erfolg vergangener und zukünftiger Migrationen dieser Art darstellt. Diese Erkenntnisse können zur Verfeinerung von Modellen über ökologische Nischenverschiebungen während Invasionen und zur Verbesserung von Vorhersagen über künftige marine Invasionen genutzt werden.

1. Introduction

1.1 Biological invasions and resulting adaptations

Ongoing climate change may influence biological invasions by altering the likelihood of introduction and establishment of alien species into new habitats (Hellmann et al., 2008; Hulme, 2017). Such processes pose major threats to biodiversity, i.e., competition, predation and hybridization with native species, modifications to ecological processes, loss of biodiversity, increase in pests and diseases (Bax et al., 2003; Crooks, 1998; Grosholz, 2002; Mack et al., 2000), and the resulting changes in the ecosystem may have serious functional and economic impacts (Simberloff et al., 2013). Therefore, to predict and mitigate future invasions it is important to understand the mechanisms that allow a species to become invasive. A successful invasive species must pass through a variety of environmental filters (e.g., Theoharides and Dukes, 2007; Vermeij, 1996; Williamson, 2006). Barriers such as geography, abiotic conditions, biotic interactions, and landscape factors structure the successive steps of the invasion (transport, colonization, establishment, landscape spread; Figure 1), and success [illegible] echanisms (Hellmann et al., 2008).

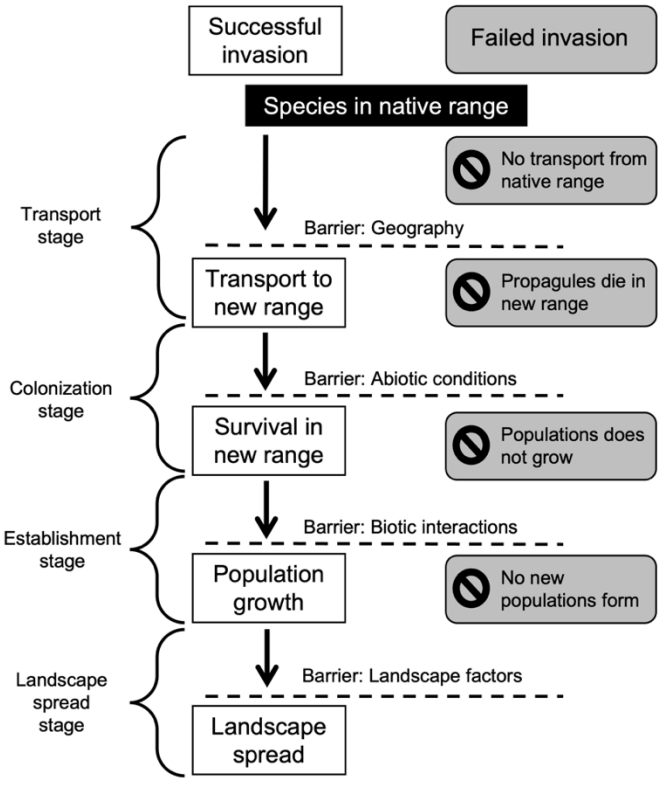


***Figure 1**: Conceptual model of the process [illegible] biological invasions, including possible successful a[illegible] failed scenarios for each stage of invasion. Adapt from Hellmann et al. (2008).*

The challenge of facing these environmental barriers and establishing a new population under foreign conditions could be counteracted by adaptations. In this scenario, successful invaders are expected to display a high adaptive potential, i.e., genetic variance needed to respond to selection (Parker et al., 2003; Weinig, 2000). A high adaptive potential could lead to the rapid selection and emergence of new variants in the invasive population (e.g., as observed in a Lessepsian cornetfish; Bernardi et al., 2016). Alternatively, the source population could already possess pre-adaptations

that allow them to thrive in the new habitat, either inherited from its evolutionary history (Davis and Shaw, 2001; de Lafontaine et al., 2018), or due to past migration events (Mimura and Aitken, 2007) or ecological filtering (Fine et al., 2013). In the case of a pre-adaptation, one might expect that the invading population could show a reduced genetic diversity compared to the source population because only a fraction of the source population participated in the invasion (founder effect, Dlugosch and Parker, 2008; Lee, 2002; Sakai et al., 2001). Either way, the increased divergence due to high adaptative potential or the reduced variability due to pre-adaptation or founder effect should exhibit a gradient along the invasion range (Quinn et al., 2000; Yue et al., 2010).

Besides a potential genetic divergence, exposure to the new conditions in the invaded area can also induce changes in microbiome associations in symbiont-bearing organisms (e.g., Howells et al., 2016). Symbiont-bearing organisms rely on their microbiome (i.e., host-associated eukaryotes and bacteria) to preserve homeostasis. Therefore, to cope with physiological stress, the host finds ways to adjust their microbiome associations, which can be highly beneficial for local adaptation (e.g., as observed in corals, Howells et al., 2016). These adaptations can occur via microbiome frequency shifts to increase the proportion of beneficial organisms (microbiome shuffling), or via acquisition of novel and serendipitous microbiome strains (microbiome switching) via horizontal transfer with the surrounding environment or between hosts (Rosenberg and Zilber-Rosenberg, 2011; Webster and Reusch, 2017). Alternatively, the invaders could already host a microbiome that possesses key adaptations (Joy, 2013; Schmidt et al., 2016a) that allows niche expansion of the holobiont (i.e., host and associated microbiome). Either way, symbiotic associations have the potential to broaden ecological niches (i.e., the set of all biotic and abiotic conditions in which a species is observed in nature), and the metabolic capabilities of host-symbiont partnerships (Cavanaugh, 1994).

However, when colonizing new habitats, instead of expanding, invasive species could be preserving or even contracting their ecological niche (Guisan and Thuiller, 2005; Pearman et al., 2008). A niche preservation could be expected when invasive species are pre-adapted to the new locations. A niche contraction could happen in the case of only a fraction of the source population surviving the numerous invasion barriers resulting in a lower genetic diversity of the host. The ecological niche is therefore an important concept for understanding patterns of species distributions (Liu et al., 2020) and adaptations to different environmental conditions (Tingley et al., 2014; Wiens et al., 2009). Most invasive species seem to conserve their climatic

niches during invasions (Liu et al., 2020). However, with climate change, one cannot predict what will be the adaptive potential of invaders.

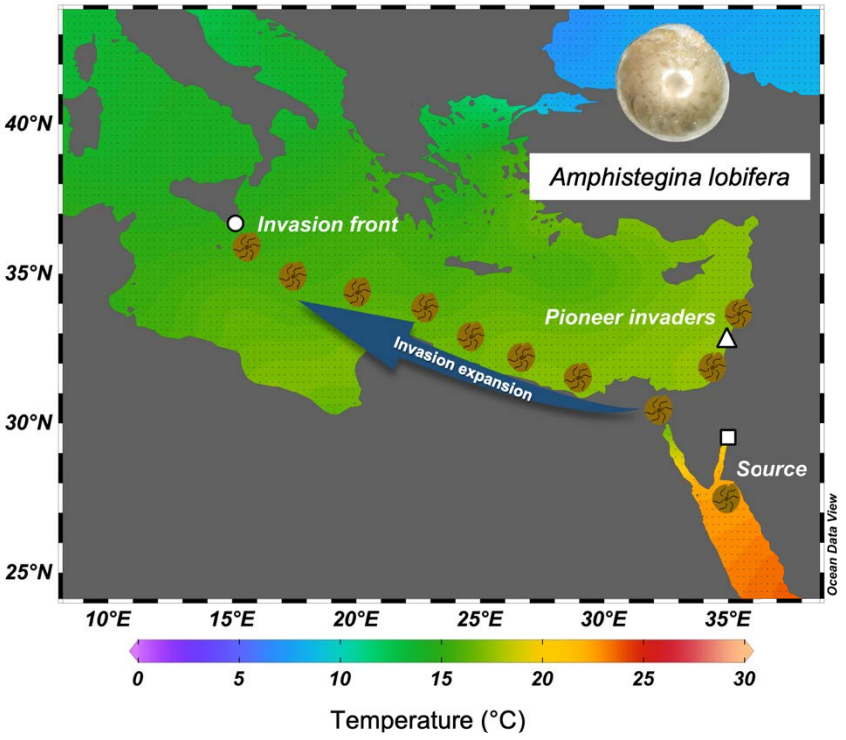
1.2 Lessepsian invasion (perfect natural laboratory)

The Mediterranean Sea serves as an ideal natural laboratory (i.e., an intermediate scale between laboratory experiments and global processes) for the invasion of marine organisms because of its semi-enclosed state that allows us to trace the development of invasions. Since the last c.a. 150 years, the Mediterranean Sea has seen a massive biological invasion of tropical species from the Red Sea known as the Lessepsian invasion. The mostly one-way invasion began with the opening of the Suez Canal in 1869, which established a migratory route from the Indo-Pacific into the Mediterranean (Rilov and Galil, 2009). Since then, the marine biota of the Eastern Mediterranean has rapidly changed as a result of the 680 invasive species that have been already documented over the past several decades, including fish, crustaceans, molluscs, and other marine animals and plants (Galil et al., 2021; Galil and Goren, 2014). Because the Levantine basin continues to warm at a rate of more than 0.1 °C/year (Ozer et al., 2017), this allows the tropical Indo-Pacific species to endure and expand farther into the Mediterranean. However, the invasion continues to expand north and west in the Mediterranean, where species encounter greater seasonality in which temperatures may reach 31°C in summer and 11 °C in winter (Borzelli and Ligi, 1999; Sorgente et al., 2003). These temperatures exceed what they experience in the Red Sea, where there is essentially no seasonal variation throughout the year (from 22 °C in winter to 28 °C in summer) (Schmidt et al., 2016a). Moreover, the minimum temperature could be a limiting factor for the expansion of the tropical species and potentially could mitigate the effect of global warming and thus counteract invasive species.

Among the particularly successful Lessepsian invaders is the symbiont-bearing larger benthic foraminifera (LBF) species *Amphistegina lobifera*. The genus *Amphistegina* is found in shallow coastal areas, attached to macroalgae, sediment biofilm, pebbles, or coral reef fragments (Langer and Hottinger, 2000). Propagules, the smallest form that foraminifera occur in throughout their life cycle, can be easily moved by currents (Alve and Goldstein, 2003), and occasionally the nearly sessile adult specimens can also be transported (Alve, 1999), resulting in a wide expansion range of this species (Prazeres et al., 2020). Their pioneer-invader populations established themselves in the Levantine basin in the 1960s (Langer, 2008), and after a few decades of progressive north and westward expansion (el Kateb et al., 2018; Langer

and Mouanga, 2016; Meriç et al., 2008; Triantaphyllou et al., 2012; Yokes et al., 2007; Figure 2), they recently reached the invasion front in Sicily (Guastella et al., 2019; Figure 2). There, the invaders cope with minimum winter temperatures of ~ 13°C (Sorgente et al., 2003), which is at least 9 °C below what they experienced in their natural habitat. Yet, in a short period after the species' first appearance in Sicily in 2016, this prolific calcifier became abundant, sometimes reaching 50% of the foraminifera assemblages (Guastella et al., 2019).

Amphistegina is a diatom-bearing LBF (Lee, 2006), being part of the 1% of 10,000 extant foraminifera species that are known to host algal symbionts (Lee and Anderson, 1991). Interestingly, the foraminifera species that developed algal symbioses often became relatively large (i.e., the LBFs, Lee and Hallock, 1987) and increasingly dominant in oligo-trophic environments (Hallock and Schlager, 1986), constituting major calcium carbonate producers in shallow coastal waters (Erez and Gill, 1977; Hallock, 1981). Therefore, the enhanced growth and calcification in these organisms are tightly related to their algal symbionts, analogous to the symbiotic associations in reef-building corals (Lee and Anderson, 1991). In fact, under heat stress, *Amphistegina* expels its symbionts (Schmidt et al., 2016b) in a process that leads to the bleaching of the host (Hallock et al., 2006; Schmidt et al., 2011). This similarity between LBF species and reef-building corals and the adaptation of both groups to nutrient-poor waters reveals that LBF can be used as a biological index for coral reef assessment (Hallock et al., 2003). In addition, foraminifera have further advantages, such as their relatively small size and large abundance, permitting statistically significant sample sizes to



Mediterranean Sea. Background color shows average sea surface temperatures in winter (Jan-Mar) for all years available on the World Ocean Atlas 2018.

be collected quickly and with minimal impact on reef resources (Hallock et al., 2003). This makes the LBF an attractive low-cost and low-impact alternative to investigate the ecological niche and the effects of range expansions in marine holobionts. Moreover, because of the fast reproduction in LBFs (Hallock, 2003) which facilitates evolutionary adaptation, and their well-documented history of invasion into the Mediterranean, these foraminifera are a suitable model for this purpose.

2. Thesis objectives and outline

2.1 Research questions

Amphistegina lobifera stands out from other tropical organisms that have not colonized the Mediterranean because it can thrive in the new habitat despite the severe winters compared to the source location in the Red Sea. Not even the sister species *Amphistegina lessonii* seems to resist the cold winter temperatures, as shown by the extremely rare occurrence of this species in the Mediterranean (Hollaus and Hottinger, 1997). This reveals the importance of temperature on species distribution (Titelboim et al., 2019) and enables the use of *A. lobifera* as a model system to explore the mechanism of cold stress tolerance in marine holobionts.

Although the large potential dispersal of *A. lobifera* is well described (Prazeres et al., 2020), as well as its potential to tolerate heat stress (Schmidt et al., 2016a; Titelboim et al., 2019), no investigation was done on the impact of low winter temperatures, such as the ones faced in the Mediterranean Sea, that were never experienced in its source location. This raises ***Research Question 1****: Is the invasion (a) facilitated by or (b) did it induce genetic adaptation of the foraminifera host or (c) did the source population sustain key pre-adaptations?* In this context, the success of invasion could be due (i) to a rapid genetic adaptation of the host, (ii) to a selection of a specific fraction of the source population, or (iii) to key pre-adaptations to the new locations in the source population with the opening of the Suez Canal just removing a physical barrier after which the invader rapidly fills the free space. The two first possibilities would increase the genetic divergence between the invasive and source populations, whereas the third option would result in no clear genetic divergence across the invasive range.

In addition to a potential genetic divergence of the host, ecosystem disturbances also have the potential to disrupt the interactions that contribute to the fitness of the host-associated microbiome (Greenspan et al., 2019). In other marine holobionts such as corals, the acquisition of new beneficial symbionts (Howells et al., 2016), including bacteria (Gilbert et al., 2012; Ziegler et al., 2017), have induced tolerance to thermal stress of the holobiont. The tolerance to heat stress of invasive *A. lobifera* diatoms is well documented (Schmidt et al., 2016a; Titelboim et al., 2019). However, their tolerance to cold stress remains largely unknown. Moreover, although the main photo-symbiotic partners in *Amphistegina* are well-known and described (i.e., Lee, 2006; Prazeres et al., 2021), only a few studies on their bacterial communities have been conducted (Prazeres, 2018; Prazeres et al., 2017). Furthermore, the flexibility of the host to form new associations with the taxa available in the surrounding

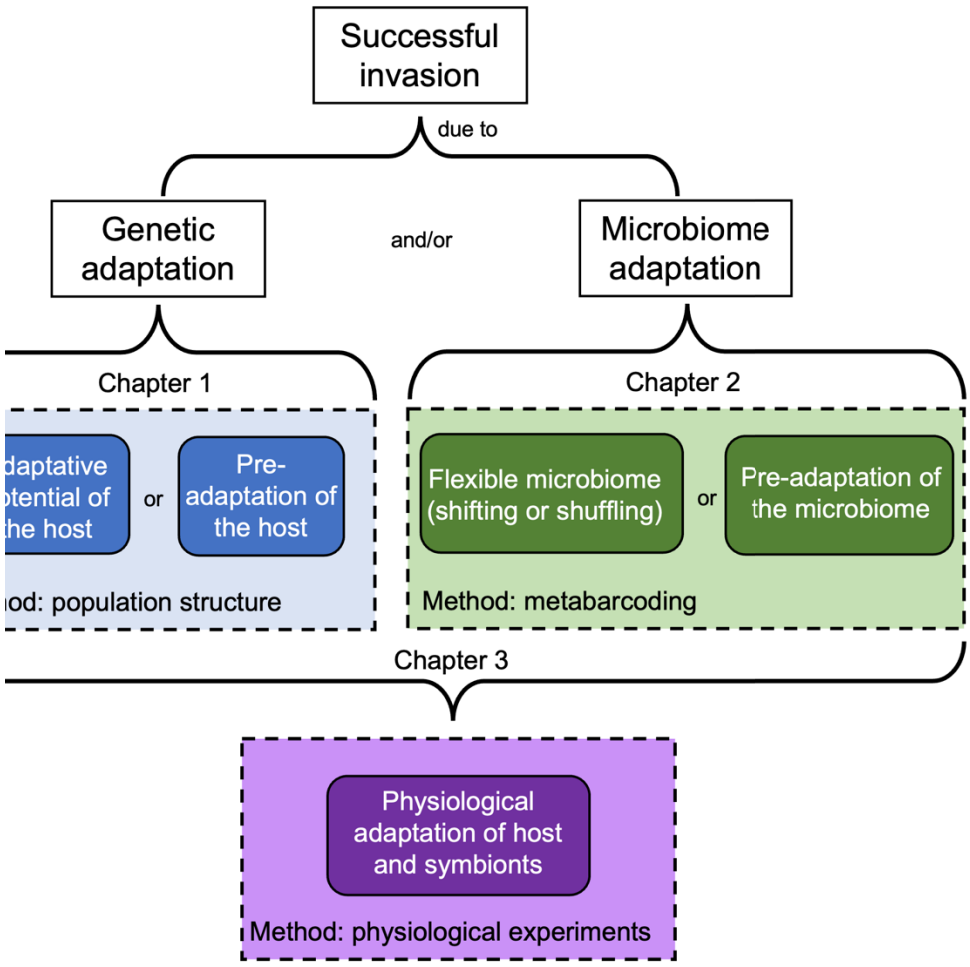
environment remains unresolved. This raises ***Research Question 2****: Is the local adaptation enhanced by beneficial microbiome associations (microalgal and bacterial) which the foraminifera acquired during invasion, or did they carry the same associations as the source population?* Answering this question is an important first step toward understanding the role of their microbiome in the invasion success and predicting future range expansions of *A. lobifera* in the Mediterranean.

Finally, regardless of the mechanism that provides the invasion success of *A. lobifera*, little is known about whether, after decades of progress in the Mediterranean, the holobiont has developed physiological adaptations to severe winter temperatures. This raises **Research question 3***: Did the sustained exposure to colder winters in the invasive locations induce adaptations in the foraminiferal host and/or its symbionts, or is there an inherited pre-adaptation from the source population?* This question, therefore, tries to answer if the adaptation necessary for the successful and rapid invasion in the Mediterranean Sea is related to a cold tolerance of the invasive foraminifera host, its symbionts, or both.

2.2 Thesis outline

The aim of this thesis is to investigate the factors behind the success of invasion of an indo-pacific larger benthic foraminifera species in the Mediterranean Sea. For this, a combination of different approaches are applied, including population genetics, metabarcoding analyses, and physiological experiments (Figure 3). This work is a cumulative thesis consisting of three manuscripts and one appendix chapter. The Chapter 1 is under consideration for publication in the journal *Scientific Reports*, and Chapters 2 and 3 are in preparation for submission. The Appendix Chapter has been published in the journal *Functional Ecology*.

The manuscript in **Chapter 1** aims to answer research question 1 whether the invasion is facilitated by a genetic adaptation of the foraminifera host. For this purpose, the population structure of invasive *A. lobifera* from the Mediterranean Sea and the source population from the Red Sea were investigated, to determine the degree of genetic divergence across the invasive range. Chapter 1 thus constrains what is the role of the foraminifera host in the success of invasion and sets the stage for Chapter 2 in which the focus is on the symbionts.



rious environmental stresses during invasion and become a successful r.

In **Chapter 2**, the research question 2 about possible flexible microbiome associations during invasion was assessed. For this, metabarcoding analyses were conducted to evaluate the eukaryotic and prokaryotic community within the foraminifera host and in the surrounding environment (sediment and seawater). The objective of these analyses was to constrain whether the invasive foraminifera carried the same microbiome as the source population or if they were able to acquire symbionts horizontally from the immediate surrounding environment. With this, Chapter 2 constrains the different eukaryotic and prokaryotic associations across the invasive range, an important piece of information before proceeding to the next research question addressed in Chapter 3 about eventual physiological adaptations in the invasive populations.

In **Chapter 3**, the main goal is to answer research question 3 whether the sustained exposure to colder winters in the invasive locations has induced adaptations in the foraminiferal holobiont, or if the foraminiferal capacity to withstand cold winters is a matter of pre-adaptation, being inherited from the source population. For this, the physiological adaptation of the host and its photosymbionts from the invasive and source populations was investigated.

Appendix Chapter: In this manuscript, the goal is to investigate the molecular response of invasive foraminifera and its photosymbionts to the thermal stress experienced in invasive locations. Differential gene expression was assessed in a controlled experiment to quantify the physiological response of the foraminifera holobiont to cold and heat stress by transcriptomic sequencing.

2.3 Own contributions to manuscripts

The fundamental concept of the first study (**Chapter 1**) was formulated by the candidate with contributions from MK and RM. She planned and organized the field trips (one in Sicily and two in Israel), collected all samples and isolated all individual foraminifera with the assistance of RM. She ensured the preservation of all samples for the subsequent analyses. Together with RM, she developed a new protocol for DNA extraction of a long segment of the rRNA gene including the small sub-unit (SSU) and the more informative internal transcribed spacer (ITS) regions, which was amplified for the first time in Amphistegina during this study. She carried out all the extractions, optimized the PCR protocol, and performed all PCRs. With the assistance of RM, she designed and conducted the cloning protocol. She picked all clones, carried out the final PCRs, and prepared samples for sequencing with an external provider. The candidate performed all sequences analyses including: quality checks of chromatograms, preparation of consensus sequences, alignment, and preparation of FASTA file and associated metadata. With help of RM, she extracted sequence data available in an online database and analyzed these sequences together with the ones generated by her. She conducted phylogenetic analyses with the assistance of RM and analyzed the patristic distances with help of CH. She compiled all data, generated all figures, and implemented all statistical analyses. She prepared the first draft of the manuscript with advice from MK and RM. The analyses benefitted from collaborations and insights from all co-authors either on the interpretation of the data or on the structure of the manuscript.

For the second study (**Chapter 2**), the candidate developed the research goals and designed the sampling scheme, jointly with MK and RM. With the assistance of RM, she planned and organized the field trips (the same as in Chapter 1), and collected all samples, including the extraction of individual foraminifera from sediment samples and sampling of environmental DNA from the sediment and by water filtration, as well as preservation of the samples for subsequent analyses. She developed an optimised protocol for DNA extraction from the foraminifera samples and conducted all DNA extractions from single cells and from the environmental samples (filters and sediment). With the help of RM, she optimised PCR protocols for the eukaryotic and bacterial amplification of the extracts and prepared all samples for metabarcoding with next-generation sequencing with external provider, including primer tagging for both eukaryotic and prokaryotic microbiomes, and PCRs. With the assistance of CH, she performed all bioinformatic analyses, including demultiplexing and filtering of raw reads, identification of amplicon sequence variants, merging forward and reverse reads, identifying chimerical sequences and conducting the taxonomical assignment. The candidate harmonised all data produced, all the figures, and designed and implemented all statistical analyses of the metabarcoding data. She interpreted the data and wrote the first draft of the manuscript with the assistance of RM and CH.

For the third study (**Chapter 3**), the candidate formulated the research goals with the support of MK and RM. She planned and organized the field trips (the same as in Chapter 1), collected all samples from which she obtained all living foraminifera with the assistance of RM. She ensured the preservation of all living samples during shipment to Bremen as well as in cultures once in the laboratory. She designed the physiological experiment with advice from CS and RM. The candidate was responsible for conducting the physiological experiment including the maintenance of temperature, light and salinity conditions, the weekly measurements of photosynthetic activity of the symbionts and movement (motility) of foraminifera, and the size measurements of the foraminifera & chlorophyll extractions both before and after the experiment. She then analysed the data and carried out the statistical analysis with advice from CH. The candidate produced all the figures, interpreted the results, and wrote the first draft of the manuscript with contributions from RM, CH and MK.

The **Appendix Chapter** has been designed by DT, SA and MK, and the candidate contributed with the results of her physiological experiment research. The candidate decided to add this appendix chapter to her dissertation by the suggestion of MK. She planned and carried out the

physiological experiment that contributed to the manuscript. She harmonized all data, prepared the figure and the corresponding statistical analysis. The candidate contributed to the manuscript writing and revision.

References

Alve, E., 1999. Colonization of new habitats by benthic foraminifera: A review. Earth Science Reviews 46, 167–185. https://doi.org/10.1016/S0012-8252(99)00016-1

Alve, E., Goldstein, S.T., 2003. Propagule transport as a key method of dispersal in benthic foraminifera (Protista). Limnol Oceanogr 48, 2163–2170. https://doi.org/10.4319/lo.2003.48.6.2163

Bax, N., Williamson, A., Aguero, M., Gonzalez, E., Geeves, W., 2003. Marine invasive alien species: A threat to global biodiversity. Mar Policy 27, 313–323. https://doi.org/10.1016/S0308-597X(03)00041-1

Bernardi, G., Azzurro, E., Golani, D., Miller, M.R., 2016. Genomic signatures of rapid adaptive evolution in the bluespotted cornetfish, a Mediterranean Lessepsian invader. Mol Ecol 25, 3384–3396. https://doi.org/10.1111/mec.13682

Borzelli, G., Ligi, R., 1999. Autocorrelation Scales of the SST Distribution and Water Masses Stratification in the Channel of Sicily. Journal of Atmospheric and Oceanic Technology 16, 776–781. https://doi.org/10.1175/1520-0426(1999)016<0776:ASOTSD>2.0.CO;2

Cavanaugh, C.M., 1994. Microbial Symbiosis: Patterns of Diversity in the Marine Environment 1. Am Zool 34, 79–89.

Crooks, J.A., 1998. Habitat alteration and community-level effects of an exotic mussel, Musculista senhousia. Mar Ecol Prog Ser 162, 137–152.

Davis, M.B., Shaw, R.G., 2001. Range shifts and adaptive responses to quaternary climate change. Science (1979) 292, 673–679. https://doi.org/10.1126/science.292.5517.673

de Lafontaine, G., Napier, J.D., Petit, R.J., Hu, F.S., 2018. Invoking adaptation to decipher the genetic legacy of past climate change. Ecology 99, 1530–1546. https://doi.org/10.1002/ecy.2382

Dlugosch, K.M., Parker, I.M., 2008. Founding events in species invasions: Genetic variation, adaptive evolution, and the role of multiple introductions. Mol Ecol 17, 431–449. https://doi.org/10.1111/j.1365-294X.2007.03538.x

el Kateb, A., Stalder, C., Stainbank, S., Fentimen, R., Spezzaferri, S., 2018. The genus Amphistegina (Benthic foraminifera): Distribution along the southern Tunisian coast. Bioinvasions Rec 7, 391–398. https://doi.org/10.3391/bir.2018.7.4.06

Erez, J., Gill, D., 1977. Multivariate Analysis of Biogenic Constituents in Recent Sediments Off Ras Burka, Gulf of Elat, Red Sea. Math Geol 9.

Fine, M., Gildor, H., Genin, A., 2013. A coral reef refuge in the Red Sea. Glob Chang Biol 19, 3640–3647. https://doi.org/10.1111/gcb.12356

Galil, B.S., Goren, M., 2014. Metamorphoses: Bioinvasions in the Mediterranean Sea BT - The Mediterranean Sea: Its history and present challenges, in: Goffredo, S., Dubinsky, Z. (Eds.), . Springer Netherlands, Dordrecht, pp. 463–478. https://doi.org/10.1007/978-94-007-6704-1_27

Galil, B.S., Mienis, H.K., Hoffman, R., Goren, M., 2021. Non-indigenous species along the Israeli Mediterranean coast: tally, policy, outlook. Hydrobiologia 848, 2011–2029. https://doi.org/10.1007/s10750-020-04420-w

Gilbert, J.A., Hill, R., Doblin, M.A., Ralph, P.J., 2012. Microbial consortia increase thermal tolerance of corals. Mar Biol 159, 1763–1771. https://doi.org/10.1007/s00227-012-1967-9

Greenspan, S.E., Lyra, M.L., Migliorini, G.H., Kersch-Becker, M.F., Bletz, M.C., Lisboa, C.S., Pontes, M.R., Ribeiro, L.P., Neely, W.J., Rezende, F., Romero, G.Q., Woodhams, D.C., Haddad, C.F.B., Toledo, L.F., Guilherme Becker, C., 2019. Arthropod-bacteria interactions influence assembly of aquatic host microbiome and pathogen defense. Proceedings of the Royal Society B: Biological Sciences 286. https://doi.org/10.1098/rspb.2019.0924

Grosholz, E., 2002. Ecological and evolutionary consequences of coastal invasions. Trends Ecol Evol 17, 22–27. https://doi.org/10.1016/S0169-5347(01)02358-8

Guastella, R., Marchini, A., Caruso, A., Cosentino, C., Evans, J., Weinmann, A.E., Langer, M.R., Mancin, N., 2019. "Hidden invaders" conquer the Sicily Channel and knock on the door of the Western Mediterranean sea. Estuar Coast Shelf Sci 225, 106234. https://doi.org/10.1016/j.ecss.2019.05.016

Guisan, A., Thuiller, W., 2005. Predicting species distribution: offering more than simple habitat models. Ecol Lett 8, 993–1009. https://doi.org/https://doi.org/10.1111/j.1461-0248.2005.00792.x

Hallock, P., 2003. Symbiont-bearing Foraminifera BT – Modern Foraminifera, in: sen Gupta, B.K. (Ed.), . Springer Netherlands, Dordrecht, pp. 123–139. https://doi.org/10.1007/0-306-48104-9_8

Hallock, P., 1981. Production of Carbonate Sediments by Selected Large Benthic Foraminifera on Two Pacific Coral Reefs. Journal of Sedimentary Research 51. https://doi.org/10.1306/212F7CB1-2B24-11D7-8648000102C1865D

Hallock, P., Lidz, B.H., Cockey-Burkhard, E.M., Donnelly, K.B., 2003. Foraminifera As Bioindicators In Coral Reef Assessment And Monitoring: The FORAM Index.

Hallock, P., Schlager, W., 1986. Nutrient Excess and the Demise of Coral Reefs and Carbonate Platforms, PALAIOS.

Hallock, P., Williams, D.E., Fisher, E.M., Toler, S.K., 2006. Bleaching in Foraminifera with Algal Symbionts: Implications for Reef Monitoring and Risk Assessment FORAMS 2006. Anuário do Instituto de Geociências -UFRJ 29, 108–128.

Hellmann, J.J., Byers, J.E., Bierwagen, B.G., Dukes, J.S., 2008. Five potential consequences of climate change for invasive species. Conservation Biology. https://doi.org/10.1111/j.1523-1739.2008.00951.x

Hollaus, S.S., Hottinger, L., 1997. Temperature dependance of endosymbiontic relationships? Evidence from the depth range of ilose ge ean Amphistegina lessonii (Foraminiferida) truncated by the thermocline. Eclogae Geologicae Helvetiae 90, 591–597.

Howells, E.J., Abrego, D., Meyer, E., Kirk, N.L., Burt, J.A., 2016. Host adaptation and unexpected symbiont partners enable reef-building corals to tolerate extreme temperatures. Glob Chang Biol 22, 2702–2714. https://doi.org/https://doi.org/10.1111/gcb.13250

Hulme, P.E., 2017. Climate change and biological invasions: evidence, expectations, and response options. Biological Reviews 92, 1297–1313. https://doi.org/10.1111/brv.12282

Joy, J.B., 2013. Symbiosis catalyses niche expansion and diversification. Proceedings of the Royal Society B: Biological Sciences 280, 20122820. https://doi.org/10.1098/rspb.2012.2820

Langer, M.R., 2008. Foraminifera from the Mediterranean and the Red Sea. Aqaba-Eilat, the Improbable GulfEnvironment, Biodiversity and Preservation. The Hebrew University Magnes Press, Jerusalem, pp. 394–415.

Langer, M.R., Hottinger, L., 2000. Biogeography of selected "larger" foraminifera. Micropaleontology 46, 105–126.

Langer, M.R., Mouanga, G.H., 2016. Invasion of amphisteginid foraminifera in the Adriatic Sea. Biol Invasions 18, 1335–1349. https://doi.org/10.1007/s10530-016-1070-0

Lee, C.E., 2002. Evolutionary genetics of invasive species. Trends Ecol Evol 17, 386–391. https://doi.org/10.1016/S0169-5347(02)02554-5

Lee, J., 2006. Algal symbiosis in larger foraminifera. Symbiosis 42, 63–75.

Lee, J.J., Anderson, O.R., 1991. Symbiosis in Foraminifera, in: Biology of Foraminifera. Academic Press, London, pp. 157–220.

Lee, J.J., Hallock, P., 1987. Algal Symbiosis as the Driving Force in the Evolution of Larger Foraminifera. Ann N Y Acad Sci 503, 330–347. https://doi.org/10.1111/j.1749-6632.1987.tb40619.x

Liu, C., Wolter, C., Xian, W., Jeschke, J.M., 2020. Most invasive species largely conserve their climatic niche. Proc Natl Acad Sci U S A 117, 23643–23651. https://doi.org/10.1073/pnas.2004289117

Mack, R.N., Simberloff, D., Mark Lonsdale, W., Evans, H., Clout, M., Bazzaz, F.A., 2000. Biotic Invasions: Causes, Epidemiology, Global Consequences, and Control. Ecological Applications 10, 689–710. https://doi.org/https://doi.org/10.1890/1051-0761(2000)010[0689:BICEGC]2.0.CO;2

Meriç, E., Avşar, N., Yokeş, M.B., 2008. Some alien foraminifers along the Aegean and southwestern coasts of Turkey. Micropaleontology 54, 307–349.

Mimura, M., Aitken, S.N., 2007. Adaptive gradients and isolation-by-distance with postglacial migration in Picea sitchensis. Heredity (Edinb) 99, 224–232. https://doi.org/10.1038/sj.hdy.6800987

Ozer, T., Gertman, I., Kress, N., Silverman, J., Herut, B., 2017. Interannual thermohaline (1979–2014) and nutrient (2002–2014) dynamics in the Levantine surface and intermediate water masses, SE Mediterranean Sea. Glob Planet Change 151, 60–67. https://doi.org/https://doi.org/10.1016/j.gloplacha.2016.04.001

Parker, I.M., Rodriguez, J., Loik, M.E., 2003. An evolutionary approach to understanding the biology of invasions: Local adaptation and general-purpose genotypes in the weed Verbascum ilose . Conservation Biology 17, 59–72. https://doi.org/10.1046/j.1523-1739.2003.02019.x

Pearman, P.B., Guisan, A., Broennimann, O., Randin, C.F., 2008. Niche dynamics in space and time. Trends Ecol Evol 23, 149–158. https://doi.org/10.1016/j.tree.2007.11.005

Prazeres, M., 2018. Bleaching-associated changes in the microbiome of large benthic foraminifera of the Great Barrier Reef, Australia. Front Microbiol 9, 1–13. https://doi.org/10.3389/fmicb.2018.02404

Prazeres, M., Ainsworth, T., Roberts, T.E., Pandolfi, J.M., Leggat, W., 2017. Symbiosis and microbiome flexibility in calcifying benthic foraminifera of the great Barrier Reef. Microbiome 5, 1–11. https://doi.org/10.1186/S40168-017-0257-7

Prazeres, M., Morard, R., Roberts, T.E., Doo, S.S., Jompa, J., Schmidt, C., Stuhr, M., Renema, W., Kucera, M., 2020. High dispersal capacity and biogeographic breaks shape the genetic diversity of a globally distributed reef-dwelling calcifier. Ecol Evol 10, 5976–5989. https://doi.org/10.1002/ece3.6335

Prazeres, M., Roberts, T.E., Ramadhani, S.F., Doo, S.S., Schmidt, C., Stuhr, M., Renema, W., 2021. Diversity and flexibility of algal symbiont community in globally distributed larger benthic foraminifera of the genus Amphistegina. BMC Microbiol 21, 1–16. https://doi.org/10.1186/s12866-021-02299-8

Quinn, T.P., Unwin, M.J., Kinnison, M.T., 2000. Evolution of temporal isolation in the wild: Genetic divergence in timing of migration and breeding by introduced chinook salmon populations. Evolution (N Y) 54, 1372–1385. https://doi.org/10.1111/j.0014-3820.2000.tb00569.x

Rilov, G., Galil, B., 2009. Marine Bioinvasions in the Mediterranean Sea – History, Distribution and Ecology 549–575. https://doi.org/10.1007/978-3-540-79236-9_31

Rosenberg, E., Zilber-Rosenberg, I., 2011. Symbiosis and development: The hologenome concept. Birth Defects Res C Embryo Today 93, 56–66. https://doi.org/https://doi.org/10.1002/bdrc.20196

Sakai, A.K., Allendorf, F.W., Holt, J.S., Lodge, M., Molofsky, J., With, K.A., Cabin, R.J., Cohen, J.E., Norman, C., Mccauley, D.E., Neil, P.O., Parker, M., Thompson, J.N., Weller, S.G., 2001. The population biology of invasive species. Annu. Rev. Ecol. Syst. 32, 305–32. https://doi.org/https://doi.org/10.1146/annurev.ecolsys.32.081501.114037

Schmidt, C., Heinz, P., Kucera, M., Uthicke, S., 2011. Temperature-induced stress leads to bleaching in larger benthic foraminifera hosting endosymbiotic diatoms. Limnol Oceanogr 56, 1587–1602. https://doi.org/10.4319/lo.2011.56.5.1587

Schmidt, C., Morard, R., Prazeres, M., Barak, H., Kucera, M., 2016ª. Retention of high thermal tolerance in the invasive foraminifera Amphistegina lobifera from the Eastern Mediterranean and the Gulf of Aqaba. Mar Biol 163. https://doi.org/10.1007/s00227-016-2998-4

Schmidt, C., Titelboim, D., Brandt, J., Herut, B., Abramovich, S., Almogi-Labin, A., Kucera, M., 2016b. Extremely heat tolerant photo-symbiosis in a shallow marine benthic foraminifera. Sci Rep 6, 1–9. https://doi.org/10.1038/srep30930

Simberloff, D., Martin, J.L., Genovesi, P., Maris, V., Wardle, D.A., Aronson, J., Courchamp, F., Galil, B., García-Berthou, E., Pascal, M., Pyšek, P., Sousa, R., Tabacchi, E., Vilà, M., 2013. Impacts of biological invasions: What's what and the way forward. Trends Ecol Evol 28, 58–66. https://doi.org/10.1016/j.tree.2012.07.013

Sorgente, R., Drago, A.F., Ribotti, A., 2003. Seasonal variability in the Central Mediterranean Sea circulation.

Theoharides, K.A., Dukes, J.S., 2007. Plant invasion across space and time: Factors affecting nonindigenous species success during four stages of invasion. New Phytologist 176, 256–273. https://doi.org/10.1111/j.1469-8137.2007.02207.x

Tingley, R., Vallinoto, M., Sequeira, F., Kearney, M.R., 2014. Realized niche shift during a global biological invasion. Proc Natl Acad Sci U S A 111, 10233–10238. https://doi.org/10.1073/pnas.1405766111

Titelboim, D., Almogi-Labin, A., Herut, B., Kucera, M., Asckenazi-Polivoda, S., Abramovich, S., 2019. Thermal tolerance and range expansion of invasive foraminifera under climate changes. Sci Rep 9, 1–5. https://doi.org/10.1038/s41598-019-40944-5

Triantaphyllou, M. v., Dimiza, M.D., Koukousioura, O., Hallock, P., 2012. Observations on the life cycle of the symbiont-bearing foraminifer Amphistegina lobifera Larsen, an invasive species in coastal ecosystems of the ilose sea (Greece, E. Mediterranean). J Foraminifer Res 42, 143–150. https://doi.org/10.2113/gsjfr.42.2.143

Vermeij, G.J., 1996. An agenda for invasion biology. Biol Conserv 78, 3–9. https://doi.org/10.1016/0006-3207(96)00013-4

Webster, N.S., Reusch, T.B.H., 2017. Microbial contributions to the persistence of coral reefs. ISME J 11, 2167–2174. https://doi.org/10.1038/ismej.2017.66

Weinig, C., 2000. Plasticity Versus Canalization : Population Differences in the Timing of Shade-Avoidance Responses Author (s): Cynthia Weinig Published by : Society for the Study of Evolution Stable URL : https://www.jstor.org/stable/2640851 Society for the Study of Ev. Evolution (N Y) 54, 441–451. https://doi.org/10.1111/j.0014-3820.2000.tb00047.x

Wiens, J.A., Stralberg, D., Jongsomjit, D., Howell, C.A., Snyder, M.A., 2009. Niches, models, and climate change: Assessing the assumptions and uncertainties. Proc Natl Acad Sci U S A 106, 19729–19736. https://doi.org/10.1073/pnas.0901639106

Williamson, M., 2006. Explaining and predicting the success of invading species at different stages of invasion. Biol Invasions 8, 1561–1568. https://doi.org/10.1007/s10530-005-5849-7

Yokes, M.B., Meric, E., Avsar, N., 2007. On the Presence of alien foraminifera Amphistegina lobifera Larsen on the coasts of the Maltese Islands. Aquat Invasions 2, 439–441. https://doi.org/10.3391/ai.2007.2.4.15

Yue, G.H., Li, J., Bai, Z., Wang, C.M., Feng, F., 2010. Genetic diversity and population structure of the invasive alien red swamp crayfish. Biol Invasions 12, 2697–2706. https://doi.org/10.1007/s10530-009-9675-1

Ziegler, M., Seneca, F.O., Yum, L.K., Palumbi, S.R., Voolstra, C.R., 2017. Bacterial community dynamics are linked to patterns of coral heat tolerance. Nat Commun 8, 14213. https://doi.org/10.1038/ncomms14213

Chapter 1

Invasion success in a Lessepsian symbiont-bearing foraminifera linked to high dispersal ability, preadaptation and suppression of sexual reproduction

This work is under consideration for publication in the journal *Scientific Reports*.

Débora S. Raposo*[1], Rebecca A. Zufall[2], Antonio Caruso[3], Danna Titelboim[4], Sigal Abramovich[5], Christiane Hassenrück[1,6], Michal Kucera[1], Raphaël Morard[1]

[1] MARUM, Center for Marine Environmental Sciences, Universität Bremen, Bremen, Germany

[2] Department of Biology and Biochemistry, University of Houston, Houston, United States

[3] Dipartimento di Scienze della Terra e del Mare, Università degli studi di Palermo, Palermo, Italy

[4] Department of Earth Sciences, University of Oxford, Oxford, United Kingdom

[5] Department of Earth and Environmental Sciences, Ben Gurion University of the Negev, Beer Sheva, Israel

[6] Department of Biological Oceanography, Leibniz Institute for Baltic Sea Research Warnemünde (IOW), Rostock-Warnemünde, Germany

Data availability: All new sequences generated in this study are publicly available at NCBI GenBank under the accession numbers OP610171-OP610543. The associated metadata generated will be available on the public repository PANGAEA Data Publisher once the paper is published, under the DOI 10.1594/PANGAEA.950041.

ABSTRACT

Among the most successful Lessepsian invaders is the symbiont-bearing benthic foraminifera *Amphistegina lobifera.* In its newly conquered habitat, this prolific calcifier and ecosystem engineer is exposed to environmental conditions that exceed the range of its native habitat. To disentangle which processes facilitated the invasion success of *A. lobifera* into the Mediterranean Sea we analyzed a ~1400 bp sequence fragment of the rRNA gene complex covering the SSU and ITS regions in populations along the invasion gradient. We observe that the invasion is not associated with genetic differentiation, but the invasive populations show a distinct suppression of intra-genomic variability among the multiple copies of the rRNA gene. We conclude that the genetic structure of the invasive populations reflects two processes: high dispersal ability of a Red Sea source population pre-adapted to Mediterranean conditions and suppression of sexual reproduction in the invader. This discovery provides a new perspective on the cost of invasion in marine protists: The success of the invasive *A. lobifera* in the Mediterranean Sea comes at the cost of abandonment of sexual reproduction.

1. INTRODUCTION

Biological invasions driven by climate change are currently profoundly modifying ecological landscapes (Bellard et al., 2016; Simberloff et al., 2013). Unlike normal range extensions, where species are largely tracking their climatic envelope, invasive species conquer entirely new spaces, with a higher probability of facing climatic (seasonality), physical (light), chemical (salinity) or biotic (microbiome and interactome) conditions that exceed the range they experienced within their native habitat. In this context, it is important to understand how a given species can become a successful invader. The challenge of exposure to foreign conditions in the newly conquered space could be counteracted by adaptations. In this scenario, successful invaders can be expected to display a high adaptive potential (Parker et al., 2003; Weinig, 2000). Alternatively, the native population could already possess the key adaptations, for example as a result of its evolutionary history (Davis and Shaw, 2001; de Lafontaine et al., 2018), past migration events (Mimura and Aitken, 2007), or ecological filtering (Fine et al., 2013).

A remarkable biological invasion phenomenon known as the Lessepsian invasion has taken place in the Mediterranean Sea since 1869. The opening of the Suez Canal in that year ignited a dramatic and largely uni-directional migration of Indo-Pacific marine species into the

Mediterranean. So far, over 600 invasive marine species have been reported in the eastern Mediterranean (Rilov and Galil, 2009; Zenetos et al., 2012), with more new invaders appearing as the ongoing warming makes the Levantine Basin more tropics-like (Shaltout and Omstedt, 2014). Among the particularly successful invaders are symbiont-bearing larger benthic foraminifera (LBF). LBFs inhabit shallow coastal waters, where they commonly live attached to algae or hard substrate (Langer and Hottinger, 2000). Foraminifera have limited capacity for active movement during life, but adult specimens can be passively suspended and carried out by currents (Alve, 1999) and the passive mobility of the minute juveniles or propagules is likely even larger (Alve and Goldstein, 2003), resulting in large species ranges and lack of regional population differentiation (Prazeres et al., 2020).

The most successful Lessepsian invader LBF is the diatom-bearing *Amphistegina lobifera*, whose prolifically calcifying invasive populations modify the sediment substrate and displace native species (Langer et al., 2012; Mouanga and Langer, 2014). After establishing itself in the Levantine Basin, its invasion towards the west accelerated in the last few decades (Caruso and Cosentino, 2014; el Kateb et al., 2018; Guastella et al., 2021; Langer et al., 2012; Langer and Mouanga, 2016; Meriç et al., 2008; Schmidt et al., 2016a; Yokes et al., 2007) and the species now expanded its range to Sicily (Guastella et al., 2019). The invasion appears to be sourced entirely from within the western Indian Ocean genotype Ia of the species (Prazeres et al., 2020), but it remains unknown how the genetic diversity of the invading populations is structured along the invasion gradient.

Theoretically, an invading population could show a reduced genetic diversity compared to the source population because only a fraction of the source population participated in the invasion (founder effect, Dlugosch and Parker, 2008; Lee, 2002; Sakai et al., 2001) or because the source population is highly structured and only one subpopulation possessed adaptations allowing it to invade (Leger and Rice, 2007; Maron et al., 2004; Ward et al., 2008). The combination of a founder effect and exposure to the new environment could also lead to rapid emergence of new variation in the invasive population (e.g., as observed in a Lessepsian cornetfish, Bernardi et al., 2016). In both cases, the reduced variability and increased divergence should show a gradient along the invasion progression (Quinn et al., 2000; Yue et al., 2010), with most severe effects visible at the invasion front. Alternatively, the source population could have already possessed the necessary adaptations to the new environment and in the presence of a large dispersal potential (Prazeres et al., 2020), the opening of the Suez Canal could have just

removed a physical barrier after which the invader rapidly fills the free space without genetic differentiation.

Here we investigated the population structure of the invasive *A. lobifera* between the source population and populations representing different stages of the invasion. The analysis is based on a ~1400 bp long sequence fragment of the rRNA gene complex covering the end of the SSU coding region and the adjacent internal transcribed spacer (ITS) region. In foraminifera, the SSU rRNA gene contains fast-evolving variable regions, which provide a resolution within species (André et al., 2014; Pawlowski and Lecroq, 2010; Pillet et al., 2012; Weber and Pawlowski, 2014) and the ITS rRNA gene region provides an even higher resolution given its higher mutation rate (Tsuchiya et al., 2008). Therefore, this marker should allow us to detect genetic differentiation (or a lack thereof) among the invasive populations of *A. lobifera* and thus constrain to what degree the invasion success of the species is due to novel adaptive change or preexisting adaptations.

2. MATERIALS AND METHODS

To characterize the genetic variability of *Amphistegina lobifera* along the invasion gradient, we sampled populations of the species in Sicily, where the most recent invasion front has been identified by Guastella et al. (2019), and in Israel where populations representative of the source population (Red Sea) and pioneer invaders (Eastern Mediterranean Sea) could be collected (Figure 1). During the sampling in Sicily in September 2019, we first carried out an exploratory survey to identify the position of the invasion front compared the observations of Guastella et al. (2019) that were done between 2015 and 2017. We focused our effort on the eastern coast of Sicily, where the invasion front was located by Guastella et al. (2019) between Capo Passero to the South and Brucoli to the North. We revisited these two locations and sampled two additional locations in between (Arenella and Plemmirio) and two additional locations North of Brucoli (Cannizzaro and Recanati), assuming that the invasion front may have moved further North since 2017. At each location, pebbles and macroalgae were collected from the depth of 0.5-5 m by snorkeling. The collected substrates were brushed, and the recovered material was sieved at 63-500 µm and transferred in 0.5 L jars that were filled with ambient seawater. We then examined the samples under a stereomicroscope and assessed qualitatively the presence of *A. lobifera* in the samples.

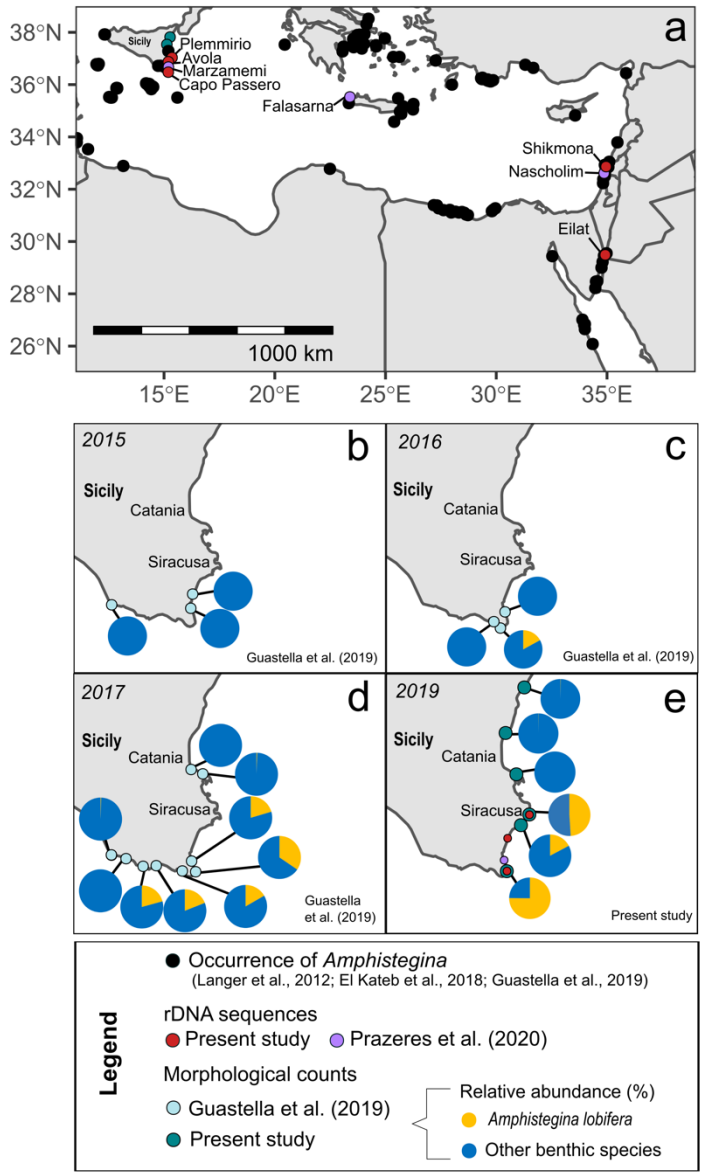


***Figure 1**. Occurrence of invasive Amphistegina in the Mediterranean Sea and sites sampled for genetic analysis (a, and e for zoom in the Sicilian sites) and progress of invasion in Sicily from 2015 until the present study assessment in 2019 (b-e)*

We observed that the species was abundant at Capo Passero, Arenella and Plemmirio but absent or rare in the samples from Brucoli, Cannizzaro and Recanati. The collected samples for the exploratory phase were air-dried and transported to our laboratory in Bremen, Germany to quantitatively assess the progress of the invasion. The dried samples were weighed and split to obtain representative aliquots containing ~300 foraminifera per sample to determine the abundance of *A. lobifera* shells in the total assemblage (living + dead) and estimate its population density (individuals/g of sediment). The results are provided in Supplementary Table S1.

Following the exploratory survey, samples for genetic analyses were collected at Capo Passero and Plemmirio and at Avola located midway. The samples were collected as described above, but now living specimens of *A. lobifera* were isolated and individually transferred to micropaleontological slides, where they were air dried and stored at -20°C (methods are detailed in Hallock et al., 2006; Schmidt et al., 2016; Stuhr et al., 2018). The isolated foraminifera were shipped on dry ice to our laboratory in Bremen, where they were stored at -80°C. The sampling in Israel was conducted in October 2019. We sampled at Shikmona, Haifa (Mediterranean Sea) and at Eilat, Gulf of Aqaba (Red Sea) where the presence of *A. lobifera* has already been documented

(Hottinger et al., 1993; Schmidt et al., 2016a; Titelboim et al., 2019). The living specimens for genetic analyses were collected and isolated and transported as described above.

Between 10 and 24 specimens per site were selected for genetic analyses. We also analyzed nine specimens of *A. lobifera* collected at Okinawa, Japan (26.651819 N; 127.856243 E) in September 2015, that were present in our collection to serve as an outgroup. Each specimen was isolated in 50 µl of DOC buffer and the thick calcite shell was cracked with a sterilized crusher. Following the DOC protocol (Weiner et al., 2016), each specimen was incubated at 60 °C for 1 hour followed by a centrifugation stage at 10.000 rpm for 5 min and stored at 4°C until further use.

Because we aimed at capturing a population dynamic process, we designed a protocol to access the most variable genomic region known for foraminifera, the Internal Transcribed Spacer (ITS) that is located between the ribosomal Small Sub-Unit (SSU) and the Large Sub-Unit (LSU). The genetic diversity of *A. lobifera* has been previously documented in the Indo-Pacific and the Mediterranean Sea based on a 600 bp fragment located at the end of the SSU (Prazeres et al. 2020). To make our results compatible with the existing data, we designed a protocol to amplify the same SSU fragment together with the entire ITS. We developed a semi-nested PCR protocol using the primers pairs S14F3 (5′-ACGCAMGTGTGAAACTTG-3′) – L5F (5′ -TCGCCGTTACTAAGGRAATC – 3’). And S14F1 (5′-AAGGGCACCACAAGAACGC-3′) – L5F(Weiner et al., 2016). The amplification was carried out using the GoTaq polymerase (Promega) with a PCR mix containing MilliQ water, 5× green buffer (final concentration: 1×), each primer (final concentration: 0.2 µmol/µl), $MgCl_2$ (final concentration: 2.5 µmol/µl), dNTP mix (final concentration: 0.4 µmol/µl) and GoTaq polymerase (final concentration: 0.05 U/µl), and added DNA extract diluted 1:10 to reduce inhibition, with a final volume of 15 µl. The second PCR was carried out with the same mix but using 1 µl of the 1st PCR as the DNA template. The thermal cycling was as follows for both successive PCRs: initial denaturation at 95°C for 2 min followed by 35 cycles of 30 s of denaturation at 95°C, annealing for 30 s at 55°C and extension at 72°C for 45 s, followed by a final extension at 72°C for 2 min.

The PCR product obtained was migrated on 1.5% agarose gel and visually checked under UV light. The samples showing single bands were selected and purified using the QIAquick PCR purification kit (QIAGEN) following the manufacturer’s instructions. Because of the presence of large intra-genomic variability among the multiple copies of the rRNA gene in foraminifera(Weber and Pawlowski, 2014), the purified PCR product was cloned using the

TOPO® TA Cloning Kit (Invitrogen, USA). Amplicons were ligated to a pCR 2.1TOPO® vector, transformed into One Shot™ TOP10 chemically competent *Escherichia coli* cells, and grown overnight on LB-agar plates containing ampicillin (100 mg/ml). Eight to 16 clones per specimen were selected and placed in 1.5 ml tubes containing 30 µl of MilliQ water and a final PCR was performed and sent for Sanger sequencing with an external provider (LGC Genomics, Berlin). Due to the long fragment targeted (~ 1400 bp), each PCR product was sequenced from both ends using the primers S14F1 and L5F. The chromatograms were carefully checked and assembled, and the resulting sequences were deposited on NCBI under the accession numbers OP610171-OP610543. In addition to the new sequences, we also recovered all available *A. lobifera* sequences and associated metadata available on NCBI that were mostly produced by Prazeres et al. (2020) and Schmidt et al. (2016). We limited our query to sequences covering the entire ~600 bp fragment of the SSU, resulting in a total of 256 sequences. All newly generated sequences and publicly available sequences are provided with associated metadata in Supplementary Table S2.

To assess to what degree the invasive populations of *A. lobifera* differ from the source Indo-Pacific populations, we constructed phylogenetic trees and compared the distribution of patristic distances among sequences in the different genetic types of the species as identified by Prazeres et al. (2020). The patristic distances were analyzed for intra-genomic variability (genetic distances among sequences within the same specimen), for population-level variability (genetic distances among sequences from different specimens occurring at the same population), for regional variability (genetic distances among sequences from specimens occurring at different populations within the same oceanic basin) and for geographical variability (genetic distances among sequences from specimens occurring in different oceanic basins). To this end, we constructed two phylogenetic inferences, one including all the sequences of the dataset but covering only the SSU fragment, and one including all the sequences generated in this study and covering the SSU and ITS fragment. For each inference, the sequences were automatically aligned with MAFFT (Katoh and Standley, 2013) and a phylogenetic tree was inferred using RaxML-NG (Kozlov et al., 2019) with 100 non-parametric bootstraps and using the substitution model TVM+I+G for the SSU alignment and the TIM2ef+I+G4 for the SSU+ITS alignment that were selected with Modeltest-NG (Darriba et al., 2020; Flouri et al., 2015). Both trees are provided in Supplementary Figure S1. After the inference, the patristic distances for the SSU tree and for the SSU+ITS tree were calculated and grouped according to the four categories of comparisons.

To identify the factors affecting intra-genomic distances within the invasive genotype, we used beta dispersion analysis based on Principle Coordinate analysis of square-root transformed patristic distances. We calculated the distance-to-centroid for each specimen for further statistical analyses (Anova and Wilcoxon test) to quantify the importance of the factors "oceanic basin" and "status of invasion" in structuring genetic diversity. The patristic distances and the statistical analyses were calculated using the packages *ape* (Paradis and Schliep, 2019) and *vegan* (Oksanen et al., 2022), respectively, from the software R 4.1.1(R Core Team, 2022).

To investigate the phylogeographic relationships among the different populations, haplotype networks were constructed for both the SSU alignment and the newly assembled SSU+ITS alignment. We constructed Median-Joining Networks (MJN) (Bandelt et al., 1999), following an algorithm analogous to that proposed by Excoffier and Smouse (Excoffier and Smouse, 1994) that first constructs Minimum Spanning Trees (MSTs) from a matrix of pairwise distances (absolute number of differences) among haplotypes and includes all possible MSTs using the parsimony criterion to infer and add missing node haplotypes to the MJN graph. We defined $\varepsilon = 0$ for a more stringent distance criterion to select the most parsimonious pathway (Leigh and Bryant, 2015). To allow the comparison of the population structure within the different genotypes, an analysis of molecular variance (AMOVA) between and within oceanic basins and populations was conducted for each genotype.

In addition to the AMOVA, we also calculated the phi-statistics (Fst) that refers to relative contributions of between-population variations to the overall genetic variation in the whole dataset. The groups were tested for adherence to neutrality (random evolution) assumptions, with Tajima's D (Tajima, 1989). Negative values of Tajima's D indicate an excess of low-frequency polymorphisms, consistent with positive directional selection or recent population expansion, whereas positive values indicate an excess of intermediate frequency polymorphism potentially due to balancing selection or population contraction (Wachowiak et al., 2011). Nucleotide diversity, number of haplotypes, and number of segregating sites were also calculated to investigate the degree of polymorphism within the genotypes. The networks and AMOVA were performed in the software PopART 1.7 (Leigh and Bryant, 2015), and the genetic diversity indexes and Tajima's D were calculated in the packages *haplotypes* (Aktas, 2020), *ape* (Paradis and Schliep, 2019), and *pegas* (Paradis, 2010), from the software R 4.1.1 (R Core Team, 2022).

3. RESULTS

Our field sampling in 2019 revealed that the invasion front of *A. lobifera* along the eastern coast of Sicily reached at least to Plemmirio, but not beyond Brucoli. At the same time, we observed that the abundance of *Amphistegina* in Sicily increased dramatically compared to the observations by Guastella et al. (2019). For instance, in Capo Passero the species represented 35% of the foraminifera in 2017 and in our sampling in 2019 the relative abundance has risen to 75%. In the other two sites where we found *A. lobifera* (Arenella and Plemmirio), the species represented 17 and 49% of the foraminifera (Figure 1, Supplementary Table S1). Thus, the invasive population has now become a major component of the assemblages on the southern coast of Sicily, but the invasion seems to have halted at the 14°C winter isotherm along the northern Sicilian coast, which is considered to represent the thermal limit for the species (Langer and Hottinger, 2000). This means that the Sicily populations sampled for genetic analyses (Figure 1) represent not only the invasion front but also an established invasive population in the South of the sampled coastal transect.

For the analysis of genetic variability of the invasive and source populations, we successfully amplified the SSU+ITS of four to 11 specimens per locality and sequenced between two to 20 clones per specimen, resulting in 373 SSU+ITS sequences from 37 specimens. For the SSU analysis, we combined the 259 sequences acquired from NCBI (Prazeres et al., 2020) with the new data, resulting in a dataset with 632 SSU sequences from 88 specimens (Supplementary Table S2). The phylogenetic tree inference conducted on the SSU fragment showed that all the newly sampled specimens represent lineage Ia as defined by Prazeres et al. (2020), confirming that the invasive population is sourced exclusively from the Red Sea, where only that genotype occurs.

In the median-joining haplotype network, 247 haplotypes and 387 segregating (polymorphic) sites were observed across the 632 sequences in the SSU alignment (Figure 2). The structure of the network was consistent with the phylogenetic tree with the four lineages being clearly separated. No further structure was observed within genotype Ia, where the invasive Mediterranean populations, the native population in the Red Sea and the Western-Indian Ocean populations share the same common haplotypes and there is no evidence for a systematic (geographical) divergence among them.

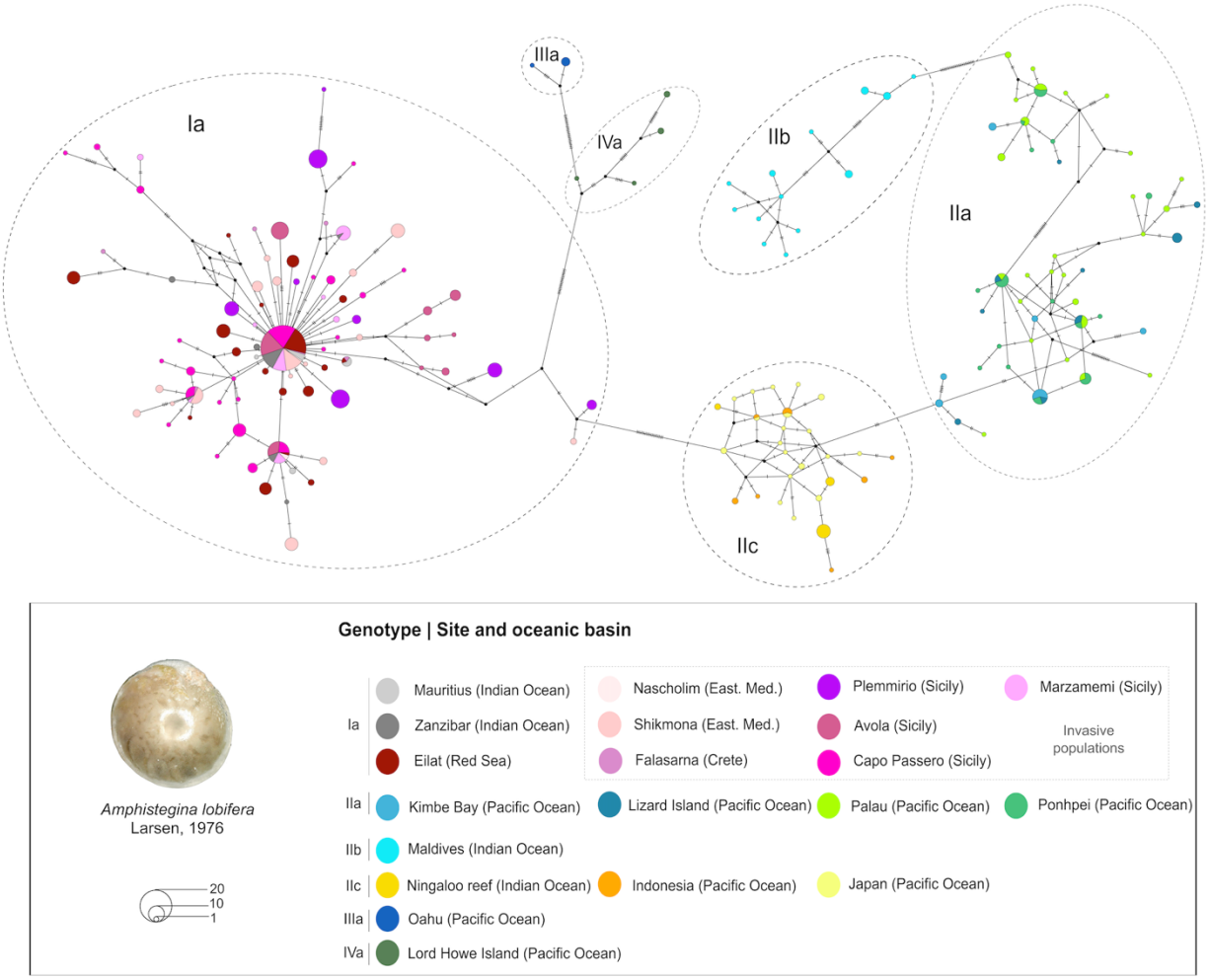


Figure 2. *Median-joining network based on SSU rRNA gene sequences of Amphistegina lobifera populations, including the invasive genotype Ia and the invasion front in Sicily*

The AMOVA of the SSU alignment revealed that within all genotypes of *A. lobifera*, the largest part of the overall genetic variation was explained by variation within populations (specimens collected from the same locality) compared to variation among oceanic basins or among populations, including within the invasive genotype Ia. The genotype Ia showed the lowest nucleotide diversity, indicating a reduced degree of polymorphism and mutation (Table 1) and it was the only genotype that showed significant deviation from neutrality (i.e., evolving randomly) based on Tajima's D (Tajima's D = -2.92, p = 0.003). The negative Tajima's D value indicates fewer haplotypes than segregating sites, which is a sign of an excess of rare alleles and can be considered an indicator of population expansion after a

Table 1. AMOVA and genetic analyses of *Amphistegina lobifera* genotypes with two or more populations (Ia, IIa and IIc)

Dataset	Alignment	Population	Oceanic basin	Sequences	AMOVA					Nucleotide diversity	Number haplotypes	Number segregating sites	Tajima's D statistic	Tajima (p)
					Variation	Df	Variation (%)	Phi st	Phi st (p) permuted					
SSU	Genotype Ia	Mauritius	West-Ind	11	Among groups	4	-8.6	0.122	**< 0.001**	0.0078	158	252	**-2.92**	**0.003**
		Zanzibar	West-Ind	23	Among Populations	5	20.8							
		Falasarna	Crete	6	Within Populations	429	87.8							
		Nascholim	East-Med	3	Total	438								
		Shikmona	East-Med	72										
		Avola	Sicily	61										
		Capo Passero	Sicily	76										
		Plemmirio	Sicily	70										
		Marzamemi	Sicily	33										
		Eilat	Red Sea	84										
		Total		439										
	Genotype IIa	Kimbe-Bay	South-Pacific	19	Among groups	1	9.9	0.133	**< 0.001**	0.031	68	149	-1.249	0.211
		Lizard-Island	South-Pacific	19	Among Populations	2	3.4							
		Palau	Pacific Ocean	41	Within Populations	107	86.7							
		Ponhpei	Pacific Ocean	32	Total	110								
		Total		111										
	Genotype IIc	Ningaloo-reef	Indian Ocean	16	Among groups	1	40.1	0.433	**< 0.001**	0.025	33	115	-1.582	0.113
		Indonesia	Pacific Ocean	11	Among Populations	1	3.2							
		Japan	Pacific Ocean	24	Within Populations	48	56.7							
		Total		51	Total	50								
SSU + ITS	Genotype Ia	Shikmona	East-Med	72	Among groups	2	-12.5	0.135	**< 0.001**	0.019	125	612	**-2.36**	**0.018**
		Avola	Sicily	61	Among Populations	2	26.0							
		Capo Passero	Sicily	76	Within Populations	356	86.5							
		Plemmirio	Sicily	70	Total	360								
		Eilat	Red Sea	82										
		Total		361										

* The negative value in the AMOVA is an artifact of the statistical approach and should be interpreted as 0%, which means that the genetic variation between the oceanic basins does not contribute to the overall total variation.

recent bottleneck or recent selective sweep (Nielsen et al., 2005; Smith and Haigh, 1974; Tajima, 1989).

To assess whether this pattern is due to genetic processes in the invasive populations of the genotype Ia, we carried out the analysis separately for sequences from the Mediterranean and from the Red Sea, and the Indian Ocean (Table 2). This revealed that the deviation from neutrality is not present in the entire genotype, but only in the Red Sea and Mediterranean populations. Finally, we carried out the analysis separately for sequences representing established invaders (Eastern Mediterranean) and the invasion front (Sicily). This revealed that both show deviation from neutrality, and it seems accentuated in the established invaders (Table 2). As expected, given the known faster mutation rate in the ITS fragment (Tsuchiya et al., 2008), the SSU+ITS haplotype network showed much higher polymorphism, with 130 haplotypes and 895 segregating sites across 373 sequences (Figure 3). The network revealed

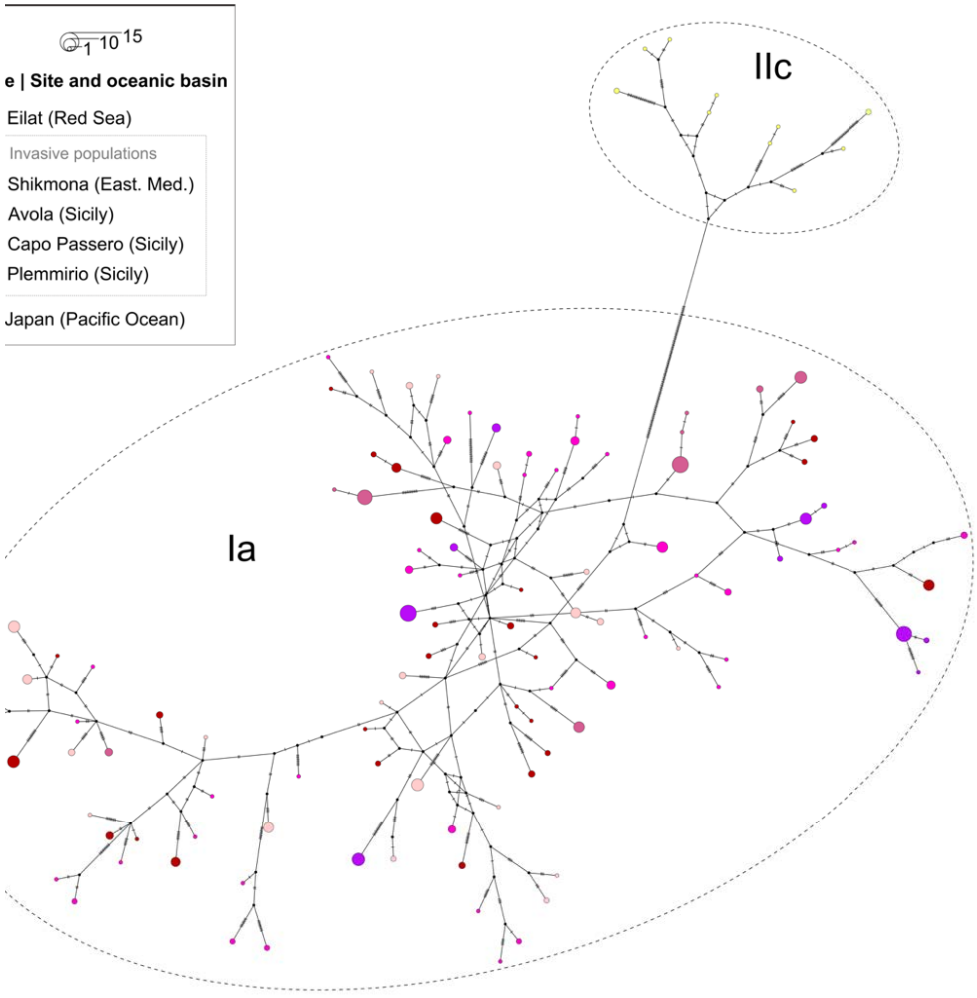


Figure 3. *Median-joining network based on SSU+ITS regions of rRNA gene sequence Amphistegina lobifera populations from invasive genotype Ia and out-group (genotype IIc)*

two main haplogroups corresponding to the invasive genotypes Ia and the outgroup genotype Iic from Japan, with neither haplogroup possessing a shared central haplotype.

Like for the SSU alignment, the AMOVA comparing the different oceanic basins within the invasive genotype Ia (Table 1) revealed that the genetic variation is higher within populations (86.5%) than among oceanic basins (-12.5%) or among populations (26.0%). The negative value in the AMOVA is an artifact of the statistical approach and should be interpreted as 0%, which means that the genetic variation between the oceanic basins does not contribute to the overall total variation at all. This is also shown by the low values of Fst (0.135) and nucleotide diversity (0.019) within genotype Ia (Table 1). Like for the SSU, the Tajima's D revealed a significant departure from neutrality (Table 1) and indicated population size expansion (e.g., after a bottleneck or a selective sweep) and this pattern is observed in both Red Sea and Mediterranean populations (Table 2). Within the Mediterranean population the deviation from neutrality is observed in both established and new invaders.

Table 2. Genetic analyses of invasive genotype Ia constrained by oceanic basins (West-Ind Ocean, Red Sea and Mediterranean Sea) and by different status of invasion in the Mediterranean (established invaders and invasion front)

Dataset	Alignment	Population	Sequences	Nucleotide diversity	Number of haplotypes	Number of segregating sites	Tajima's D statistic	Tajima (p)
SSU	West-Ind Ocean	Mauritius	11	0.024	20	105	-1.81	0.0702
		Zanzibar	23					
	Red Sea	Eilat	84	0.005	22	279	**-3.38**	**0.0007**
	Med Sea (all)	Falasarna	6	0.010	91	513	**-3.05**	**0.0023**
		Nascholim	3					
		Shikmona	72					
		Avola	61					
		Capo Passero	76					
		Plemmirio	70					
		Marzamemi	33					
	Med Sea (established invaders)	Falasarna	6	0.007	25	465	**-3.42**	**0.0006**
		Nascholim	3					
		Shikmona	72					
	Med Sea (invasion front)	Avola	61	0.011	63	202	**-2.62**	**0.0087**
		Capo Passero	76					
		Plemmirio	70					
		Marzamemi	33					
SSU + ITS	Red Sea	Eilat	82	0.018	29	420	**-2.60**	**0.0093**
	Med Sea (all)	Shikmona	72	0.019	97	535	**-2.34**	**0.0193**
		Avola	61					
		Capo Passero	76					
		Plemmirio	70					
	Med Sea	Shikmona	72	0.017	22	365	**-2.60**	**0.0095**
	Med Sea (invasion front)	Avola	61	0.019	72	477	**-2.31**	**0.0202**
		Capo Passero	76					
		Plemmirio	70					

The lack of geographical structure in the haplotype networks is reflected by the distribution of patristic distances among sequences (Figure 4). The patristic distances calculated to determine the amount of variability at the intra-genomic level (different clones from same specimen), at the population level (different specimens from same population), at the regional level (different populations from same oceanic basin) and at the geographical level (different oceanic basins) revealed little evidence for an increase in genetic divergence with geographical distance within the44ilose44ge genotype Ia. The pattern appeared both in the SSU and SSU+ITS (Figure 4a) analyses, although in the latter the distances were lower.

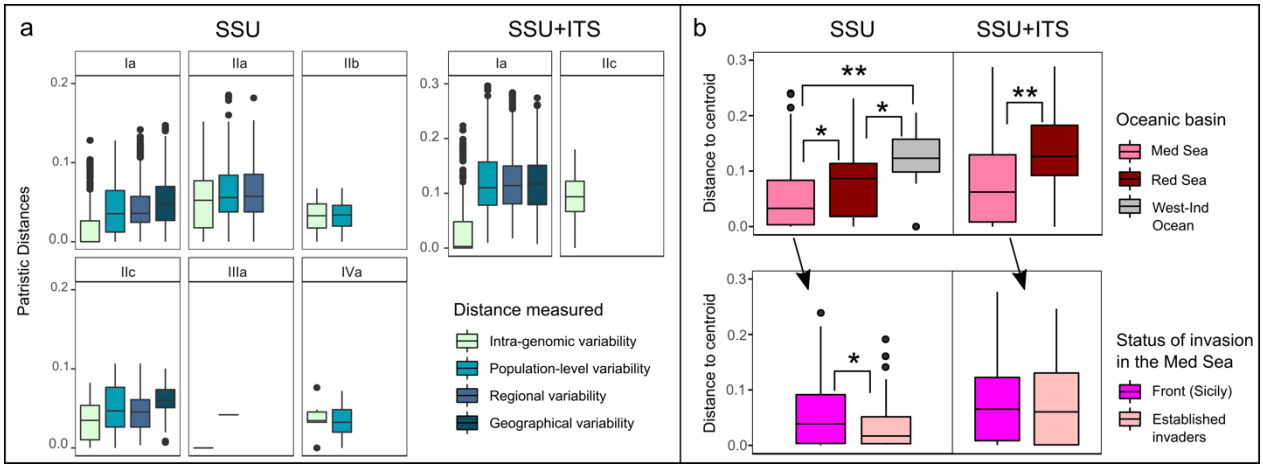


Figure 4. *Patristic distances calculated based on phylogenetic trees for the SSU and SSU+ITS rRNA gene sequences of Amphistegina lobifera including all genotypes (a) and beta dispersion analysis of intra-genomic distances within invasive genotype Ia: by oceanic basin (b, top panel) and by status of invasion in the Mediterranean Sea (b, bottom panel). Stars represent levels of significance in the Wilcoxon test shown in Table 3*

The most striking pattern revealed by the patristic distances for both the SSU and the SSU+ITS data is the large reduction of intra-genomic variability in the invasive genotype Ia (Figure 4a). This observation is highlighted when the invasive genotype Ia is compared to the other genotypes, none of which shows such a large reduction in the intra-genomic variability, except for the genotype IIIa that has too few sequences. To characterize the nature of this apparent reduction in intra-genomic variability, we compared the intra-genomic distance to centroid in the beta dispersion analysis in different populations of the invasive genotype Ia (Figure 4b and Table 3).

Table 3. ANOVA to test for factors affecting intra-genomic variability within invasive genotype (Ia) of *Amphistegina lobifera* and Wilcoxon test for pairwise comparisons of different oceanic basins and different status of invasion

ANOVA				Wilcoxon pairwise test					Level of significance
Data	Factor	Df	R2	Comparison	Group 1	Group 2	p	p.adj	
SSU (Ia)	Oceanic basin	2	0.144	Oceanic basins	West-Ind Ocean	Med Sea	1.23E-11	3.69E-11	**
	Population	7	0.119		West-Ind Ocean	Red Sea	3.30E-03	3.30E-03	*
	Specimen	43	0.520		Med Sea	Red Sea	1.42E-04	2.13E-04	*
	Residuals	386	0.218						
SSU (Ia - Med Sea)	Status of invasion	1	0.018	Status of invasion	Med Sea (invasion front)	Med Sea (established invaders)	3.44E-02	3.44E-02	*
	Population	5	0.193						
	Specimen	30	0.564						
	Residuals	284	0.224						
SSU+ITS (Ia)	Oceanic basin	1	0.092	Oceanic basins	Med Sea	Red Sea	2.00E-08	2.00E-08	**
	Population	3	0.109						
	Specimen	31	0.502						
	Residuals	324	0.297						
SSU+ITS (Ia - Med Sea)	Status of invasion	1	0.004	Status of invasion	-	-	-	-	
	Population	2	0.158						
	Specimen	23	0.546						
	Residuals	251	0.292						

This analysis reveals that the large reduction in the intra-genomic distance is present in populations from the Red Sea and significantly accentuated in the Mediterranean, both for the SSU and SSU+ITS (Figure 4b). The significant reduction in the intra-genomic distances in the Mediterranean is observed both among the established invaders and in populations at the invasion front in Sicily (Figure 4b). The degree of reduction is similar for all invasive populations with the small differences indicated in the SSU dataset not confirmed by the more informative SSU+ITS analysis (Figure 4b).

4. DISCUSSION

The observed lack of genetic difference between the invasive Mediterranean populations and the source population in the Red Sea (Figures 2 and 3) confirms the postulated large dispersal potential of *A. lobifera* (Prazeres et al., 2020). It also indicates that the invasion must have involved many of the genotypes present in the Red Sea population, rather than a hypothetical pre-adapted subtype. This means that any adaptation facilitating the invasion success was already present in the source population and the opening of the Suez Canal represented an artificial removal of an obstacle for a population that would have otherwise been able to expand beyond the Red Sea. At the same time, there does not seem to be any evidence for genetic differentiation of the invasive populations. Either the time since the invasion has been too short for unique mutations to accumulate, but this is unlikely considering the observed high within-population variability that demonstrates that the ITS is a suitable marker to capture such a

process. Or, instead, it is possible that genetic differentiation in the Mediterranean is counteracted by continuous re-seeding by populations from the Red Sea.

At the same time, we observe that the invasive and Red Sea populations show reduced genetic diversity compared to the Indian Ocean populations of the genotype Ia (Table 2). This pattern is consistent with a genetic bottleneck, which is an expected phenomenon associated with invasion (e.g., Meimberg et al., 2006; Xue et al., 2018; Zepeda-Paulo et al., 2016). Alternatively, the lower sequence diversity could also be a consequence of a selective sweep (Nielsen et al., 2005; Smith and Haigh, 1974). In this scenario, the reduced diversity in the observed marker could signal strong positive selection against another allele located in the proximity, indicating that the invasion could be associated with the presence of some particularly favorable traits. However, the pattern of low nucleotide diversity and high polymorphism is observed already in the source population from the Red Sea. This would imply that the observed bottleneck or selective sweep already affected the Red Sea population and was not associated with the Lessepsian invasion. Indeed, during each glacial lowstand, the Red Sea becomes hypersaline and inhospitable to most marine organisms, and the basin is repopulated during each deglaciation from the Indian Ocean (Fenton et al., 2000), with the last such event dating to about 11,000 years ago (e.g., Hemleben et al., 1996). The observed reduced genetic diversity could be the result of this last population expansion.

4.1 Suppression of sexual reproduction in the Mediterranean populations

Surprisingly, rather than a signal of genetic differentiation within the invading populations, we observe a strong and significant reduction of gene-copy variability throughout the Mediterranean populations (Figure 4; Table 3), which is clearly stronger than in the source population from the Red Sea. This suppression of variability between copy variants in the invasive populations is associated with the retention of high genetic variation among specimens of the same population, requiring an explanation which reduces variability within a genome but not among individuals. The only alternative that explains both phenomena is a change in the reproductive strategy towards the suppression of meiotic recombination. This is because sexual reproduction with recombination would be expected to promote genetic variation both among and within individuals. Therefore, we conclude that the observed reduction in intra-genomic variability must be a consequence of a shift towards an asexual reproductive mode that favors gene conversion-like processes leading to a loss of heterozygosity (e.g., as observed

in *Daphnia* by Tucker et al. (2013) and in *Trypanosoma* by Weir et al. (2016). Such gene conversion processes during asexual reproduction could lead to homogenization within a genome. In the presence of continuous re-seeding of the invasive populations from the Red Sea, the large variability among individuals would be preserved. Therefore, the genetic structure of the invasive population seems to reflect two processes: high dispersal potential of a pre-adapted Red Sea source population and a suppression of sexual reproduction.

Like many other foraminifera *Amphistegina* is known to have a trimorphic life cycle involving a sexual generation (agamont) and two asexual generations (gamont and schizont), with no necessity for strict alternation between asexual and sexual generations (e.g., Beavington-Penney and Racey, 2004; Dettmering et al., 1998; Hallock and Seddighi, 2020; Harney et al., 1998; Hollaus and Hottinger, 1997; Hottinger, 1982; Reiss and Hottinger, 1984). In symbiotic organisms, a change in reproduction strategy can also be linked to the process of obtaining and maintaining symbiosis. Like in many other symbiont-bearing organisms, in LBFs, asexual reproduction is associated with the vertical transfer of symbionts to the offspring. An offspring generated by multiple fission has a large size (~ 40-50 µm) and can receive the cytoplasm of the parent together with the symbionts (Dettmering et al., 1998). On the other hand, the tiny gametes (2-3 µm) cannot carry the symbionts and therefore the zygotes must acquire them from the environment. As a result, in cyclic schizogony (i.e., no alternation with sexual reproduction), the symbiont culture is maintained without the need to receive new symbionts from the environment (Dettmering et al., 1998). This could provide an explanation for the lack of sexual reproduction in the invasive *A. lobifera*. In this scenario, the ability to reproduce by cyclic schizogony would represent an advantage, or even a prerequisite, for populating regions with environmental conditions that do not allow the acquisition of new symbionts (Beavington-Penney and Racey, 2004).

Interestingly, the slightly but significantly reduced intra-genomic variability in the Red Sea population indicates that the same process, but to a lesser degree, may act already on the northern Red Sea. Compared to the Indian Ocean range of the species, the northern Red Sea already represents thermal conditions close to the limit of the species range. However, the Red-Sea populations possess tolerance to high temperatures (Schmidt et al., 2016a). Like in northern Red Sea corals (Fine et al., 2013), the retention of high tolerance in *A. lobifera* is likely the result of thermal filtering of Indian Ocean populations entering the Red Sea from the south. Therefore, it is possible that already the northern Red Sea populations of *A. lobifera* live under stressful environmental conditions that lead to partial suppression of sexual reproduction.

There are numerous observations of shifts in reproductive strategy in association with biological invasions, with most examples known among plants (e.g., Liu et al., 2006; Maurer and Zedler, 2002; You et al., 2013). That marine invasion may impede sexual reproduction among protists has not been documented before. This is significant because sex has an obvious long-term advantage by creating new combinations of genes that allow adaptation to future changing conditions (de Meeûs et al., 2007). In contrast, asexual reproduction can be an effective strategy to rapidly increase population size during the colonization of new areas (Harney et al., 1998) but has the disadvantage of decreasing the ability of the invasive population to react to future change by adaptation. Therefore, if the invasive *Amphistegina* is unable to reproduce sexually in the Mediterranean, its future proliferation may be affected by the loss of adaptive potential in the face of the projected continued environmental changes in their newly conquered space (Lejeusne et al., 2010).

5. CONCLUSION

Our results revealed that the invasion of the symbiont-bearing foraminifera *Amphistegina lobifera* in the Mediterranean Sea is facilitated by the combination of preadaptation and a high dispersal ability, with sustained re-seeding of the Mediterranean from the Red Sea. The invasion involves many of the genotypes present in the Red Sea population, rather than a specific subtype, indicating that the preadaptation to invasion was widespread in the source population. At the same time, the invasive populations show reduced intragenomic variability associated with sustained high genetic variation among specimens, which can be explained by a lower average heterozygosity due to increased gene conversion during asexual reproduction. The invasion therefore appears to be associated with a sustained change in reproductive strategy towards the abandonment of sex. Either the sexual reproduction is not triggered or cannot be completed due to adverse environmental conditions in the new habitat or, alternatively, because the zygotes have difficulty in acquiring symbionts from the environment. Either way, this discovery provides a new perspective on the cost of invasion in marine protists. If the invasion is facilitated by or requires a shift towards cyclic schizogony, the short-term gain of invasion into new habitats may be offset by a long-term loss of adaptive potential.

Data availability

The dataset with sequences and associated metadata generated during the current study are available in Supplementary Table S2 and in the NCBI repository, under the accession numbers OP610171-OP610543.

Acknowledgments

We wish to thank Dr. Giulia Visconti, director of the Plemmirio marine reserve. Sampling was possible thanks to the agreement with the Dipartimento di Scienze della Terra e del Mare (DiSTeM) of Palermo University. We thank Israel Nature and Parks Authority to have supported both sampling campaigns that were conducted in Israel under the permit 42055/2018, and Dr. Miguel Frada who kindly provided lab access at the IUI biological station. This study was funded by the BMBF-MOST cooperation in Marine Sciences Grant No. 03F0820A "ForaInva", and by the Cluster of Excellence "The Ocean Floor—Earth's Uncharted Interface" funded by the German Research Foundation (DFG).

Additional Information

Ethics declarations

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

All authors contributed to the writing and discussion of the study. Material preparation and data collection were performed by DSR, DT, SA, and RM. Data analysis was performed by DSR, CH, MK, and RM. All authors read and approved the final manuscript.

Supplementary information:

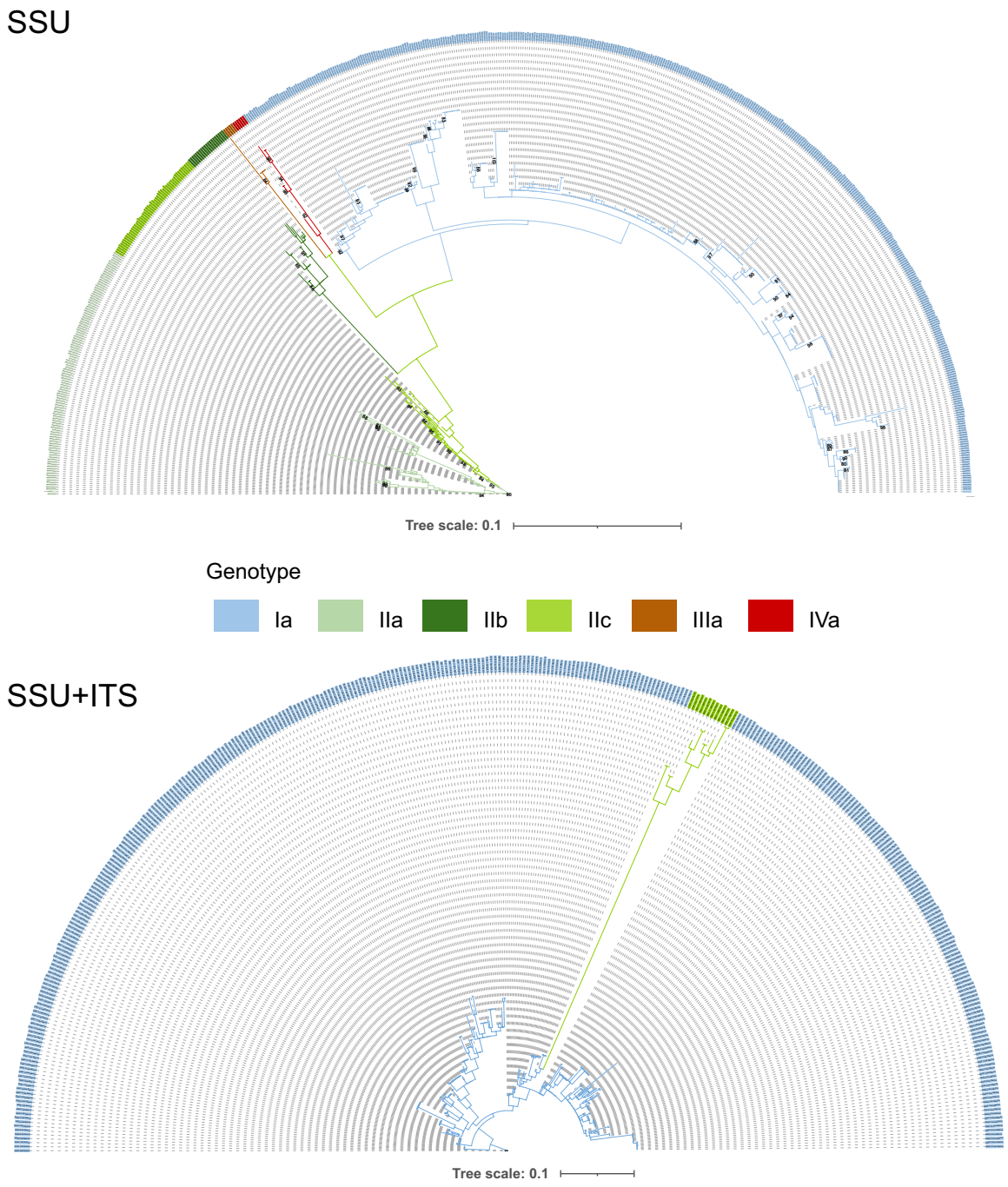


Figure S1. *Phylogenetic trees based on the SSU and the SSU+IST regions of rRNA gene sequences of Amphistegina lobifera populations for all genotypes, including the invasive genotype Ia.*

Table S1. Count of Amphistegina lobifera specimens obtained from the total assemblage (living + dead) in dried sediment samples from macroalgae and pebbles. Relative abundance of *A. lobifera* based on the population density (individuals/g of sediment) compared to the other benthic foraminifera species identified.

(Only available in the digital version of this dissertation)

Table S2. Amphistegina lobifera sequences from the SSU rRNA gene (Schmidt et al., 2016; Prazeres et al., 2020) and SSU+ITS rRNA gene (newly generated in the present study), and metadata associated.

(Only available in the digital version of this dissertation)

References

Aktas, C., 2020. Haplotypes: Manipulating DNA Sequences and Estimating Unambiguous Haplotype Network with Statistical Parsimony. R package version 1.1.2. URL https://cran.r-project.org/package=haplotypes.

Alve, E., 1999. Colonization of new habitats by benthic foraminifera: A review. Earth Science Reviews 46, 167–185. https://doi.org/10.1016/S0012-8252(99)00016-1

Alve, E., Goldstein, S.T., 2003. Propagule transport as a key method of dispersal in benthic foraminifera (Protista). Limnol Oceanogr 48, 2163–2170. https://doi.org/10.4319/lo.2003.48.6.2163

André, A., Quillévéré, F., Morard, R., Ujiié, Y., Escarguel, G., de Vargas, C., de Garidel-Thoron, T., Douady, C.J., 2014. SSU rDNA divergence in planktonic foraminifera: Molecular taxonomy and biogeographic implications. PloS One 9. https://doi.org/10.1371/journal.pone.0104641

Bandelt, H.J., Forster, P., Röhl, A., 1999. Median-joining networks for inferring intraspecific phylogenies. Mol Biol Evol 16, 37–48. https://doi.org/10.1093/oxfordjournals.molbev.a026036

Beavington-Penney, S.J., Racey, A., 2004. Ecology of extant nummulitids and other larger benthic foraminifera: Applications in palaeoenvironmental analysis. Earth Sci Rev 67, 219–265. https://doi.org/10.1016/j.earscirev.2004.02.005

Bellard, C., Cassey, P., Blackburn, T.M., 2016. Alien species as a driver of recent extinctions. Biol Lett 12. https://doi.org/10.1098/rsbl.2015.0623

Bernardi, G., Azzurro, E., Golani, D., Miller, M.R., 2016. Genomic signatures of rapid adaptive evolution in the bluespotted cornetfish, a Mediterranean Lessepsian invader. Mol Ecol 25, 3384–3396. https://doi.org/10.1111/mec.13682

Caruso, A., Cosentino, C., 2014. The first colonization of the Genus Amphistegina and other exotic benthic foraminifera of the Pelagian Islands and south-eastern Sicily (central Mediterranean Sea). Mar Micropaleontol 111, 38–52. https://doi.org/10.1016/j.marmicro.2014.05.002

Darriba, Di., Posada, D., Kozlov, A.M., Stamatakis, A., Morel, B., Flouri, T., 2020. ModelTest-NG: A New and Scalable Tool for the Selection of DNA and Protein Evolutionary Models. Mol Biol Evol 37, 291–294. https://doi.org/10.1093/molbev/msz189

Davis, M.B., Shaw, R.G., 2001. Range shifts and adaptive responses to quaternary climate change. Science (1979) 292, 673–679. https://doi.org/10.1126/science.292.5517.673

de Lafontaine, G., Napier, J.D., Petit, R.J., Hu, F.S., 2018. Invoking adaptation to decipher the genetic legacy of past climate change. Ecology 99, 1530–1546. https://doi.org/10.1002/ecy.2382

de Meeûs, T., Prugnolle, F., Agnew, P., 2007. Asexual reproduction: Genetics and evolutionary aspects. Cellular and Molecular Life Sciences 64, 1355–1372. https://doi.org/10.1007/s00018-007-6515-2

Dettmering, C., Rottger, R., Hohenegger, J., Schmaljohann, R., 1998. The trimorphic life cycle in foraminifera: Observations from cultures allow new evaluation. Eur J Protistol 34, 363–368. https://doi.org/10.1016/S0932-4739(98)80004-7

Dlugosch, K.M., Parker, I.M., 2008. Founding events in species invasions: Genetic variation, adaptive evolution, and the role of multiple introductions. Mol Ecol 17, 431–449. https://doi.org/10.1111/j.1365-294X.2007.03538.x

el Kateb, A., Stalder, C., Stainbank, S., Fentimen, R., Spezzaferri, S., 2018. The genus Amphistegina (Benthic foraminifera): Distribution along the southern Tunisian coast. Bioinvasions Rec 7, 391–398. https://doi.org/10.3391/bir.2018.7.4.06

Excoffier, L., Smouse, P.E., 1994. Using allele frequencies and geographic subdivision to reconstruct gene trees within a species: Molecular variance parsimony. Genetics 136, 343–359. https://doi.org/10.1093/genetics/136.1.343

Fenton, M., Geiselhart, S., Rohling, E.J., Hemleben, C., 2000. Aplanktonic zones in the Red Sea. Mar Micropaleontol 40, 277–294. https://doi.org/10.1016/S0377-8398(00)00042-6

Fine, M., Gildor, H., Genin, A., 2013. A coral reef refuge in the Red Sea. Glob Chang Biol 19, 3640–3647. https://doi.org/10.1111/gcb.12356

Flouri, T., Izquierdo-Carrasco, F., Darriba, D., Aberer, A.J., Nguyen, L.T., Minh, B.Q., von Haeseler, A., Stamatakis, A., 2015. The phylogenetic likelihood library. Syst Biol 64, 356–362. https://doi.org/10.1093/sysbio/syu084

Guastella, R., Marchini, A., Caruso, A., Cosentino, C., Evans, J., Weinmann, A.E., Langer, M.R., Mancin, N., 2019. "Hidden invaders" conquer the Sicily Channel and knock on the door of the Western Mediterranean sea. Estuar Coast Shelf Sci 225, 106234. https://doi.org/10.1016/j.ecss.2019.05.016

Guastella, R., Marchini, A., Caruso, A., Evans, J., Cobianchi, M., Cosentino, C., Langone, L., Lecci, R., Mancin, N., 2021. Reconstructing Bioinvasion Dynamics Through Micropaleontologic Analysis Highlights the Role of Temperature Change as a Driver of Alien Foraminifera Invasion. Front Mar Sci 8, 1–15. https://doi.org/10.3389/fmars.2021.675807

Hallock, P., Seddighi, M., 2020. Why did some larger benthic foraminifera become so large and flat? Sedimentology. https://doi.org/10.1111/sed.12837

Hallock, P., Williams, S.K., Toler, E.M., Fisher, Talge, H.K., 2006. Bleaching in reef-dwelling foraminifers: implications for reef decline. 10th Int. Coral Reef Symp 737, 729–737.

Harney, J.N., Hallock, P., Talge, H.K., 1998. Observations on a trimorphic life cycle in Amphistegina ilose populations from the Florida keys. J Foraminifer Res 28, 141–147. https://doi.org/10.2113/gsjfr.28.2.141

Hemleben, C., Meischner, D., Zahn, R., Almogi-Labin, A., Erlenkeuser, H., Hiller, B., 1996. Three hundred eighty thousand year long stable isotope and faunal records from the Red Sea: Influence of global sea level change on hydrography. Paleoceanography 11, 147–156. https://doi.org/https://doi.org/10.1029/95PA03838

Hollaus, S.S., Hottinger, L., 1997. Temperature dependance of endosymbiontic relationships? Evidence from the depth range of ilose ge ean Amphistegina lessonii (Foraminiferida) truncated by the thermocline. Eclogae Geologicae Helvetiae 90, 591–597.

Hottinger, L., 1982. Larger foraminifera, giant cells with a historical background. Naturwissenschaften 69, 361–371. https://doi.org/10.1007/BF00396687

Hottinger, L., Halicz, E., Reiss, Z., 1993. Recent foraminiferida from the Gulf of Aqaba, Red Sea, 33rd ed. Dela SAZU, Ljubljana.

Katoh, K., Standley, D.M., 2013. MAFFT multiple sequence alignment software version 7: Improvements in performance and usability. Mol Biol Evol 30, 772–780. https://doi.org/10.1093/molbev/mst010

Kozlov, A.M., Darriba, D., Flouri, T., Morel, B., Stamatakis, A., 2019. RaxML-NG: A fast, scalable and user-friendly tool for maximum likelihood phylogenetic inference. Bioinformatics 35, 4453–4455. https://doi.org/10.1093/bioinformatics/btz305

Langer, M.R., Hottinger, L., 2000. Biogeography of selected "larger" foraminifera. Micropaleontology 46, 105–126.

Langer, M.R., Mouanga, G.H., 2016. Invasion of amphisteginid foraminifera in the Adriatic Sea. Biol Invasions 18, 1335–1349. https://doi.org/10.1007/s10530-016-1070-0

Langer, M.R., Weinman, A.E., Lotters, S., Rodder, D., 2012. '"Strangers"' in Paradise: Modeling the Biogeographic Range. J Foraminifer Res 42, 234–244.

Lee, C.E., 2002. Evolutionary genetics of invasive species. Trends Ecol Evol 17, 386–391. https://doi.org/10.1016/S0169-5347(02)02554-5

Leger, E.A., Rice, K.J., 2007. Assessing the speed and predictability of local adaptation in invasive California poppies (Eschscholzia californica). J Evol Biol 20, 1090–1103. https://doi.org/10.1111/j.1420-9101.2006.01292.x

Leigh, J.W., Bryant, D., 2015. POPART: Full-feature software for haplotype network construction. Methods Ecol Evol 6, 1110–1116. https://doi.org/10.1111/2041-210X.12410

Lejeusne, C., Chevaldonné, P., Pergent-Martini, C., Boudouresque, C.F., Pérez, T., 2010. Climate change effects on a miniature ocean: the highly diverse, highly impacted Mediterranean Sea. Trends Ecol Evol 25, 250–260. https://doi.org/10.1016/j.tree.2009.10.009

Liu, J., Dong, M., Miao, S.L., Li, Z.Y., Song, M.H., Wang, R.Q., 2006. Invasive alien plants in China: Role of clonality and geographical origin. Biol Invasions 8, 1461–1470. https://doi.org/10.1007/s10530-005-5838-x

Maron, J.L., Vilà, M., Bommarco, R., Elmendorf, S., Beardsley, P., 2004. Rapid evolution of an invasive plant. Ecol Monogr 74, 261–280. https://doi.org/10.1890/03-4027

Maurer, D.A., Zedler, J.B., 2002. Differential invasion of a wetland grass explained by tests of nutrients and light availability on establishment and clonal growth. Oecologia 131, 279–288. https://doi.org/10.1007/s00442-002-0886-8

Meimberg, H., Hammond, J.I., Jorgensen, C.M., Park, T.W., Gerlach, J.D., Rice, K.J., McKay, J.K., 2006. Molecular evidence for an extreme genetic bottleneck during introduction of an invading grass to California. Biol Invasions 8, 1355–1366. https://doi.org/10.1007/s10530-005-2463-7

Meriç, E., Avşar, N., Yokeş, M.B., 2008. Some alien foraminifers along the Aegean and southwestern coasts of Turkey. Micropaleontology 54, 307–349.

Mimura, M., Aitken, S.N., 2007. Adaptive gradients and isolation-by-distance with postglacial migration in Picea sitchensis. Heredity (Edinb) 99, 224–232. https://doi.org/10.1038/sj.hdy.6800987

Mouanga, G.H., Langer, M.R., 2014. At the front of expanding ranges: Shifting community structures at amphisteginid species range margins in the ilose ge ean sea. Neues Jahrb Geol Palaontol Abh 271, 141–150. https://doi.org/10.1127/0077-7749/2014/0381

Nielsen, R., Williamson, S., Kim, Y., Hubisz, M.J., Clark, A.G., Bustamante, C., 2005. Genomic scans for selective sweeps using SNP data. Genome Res 15, 1566–1575. https://doi.org/10.1101/gr.4252305

Oksanen, J., Simpson, G., Blanchet, F., Kindt, R., Legendre, P., Minchin, P., O'Hara, R., Solymos, P., Stevens, M., Szoecs, E., Wagner, H., Barbour, M., Bedward, M., Bolker, B., Borcard, D., Carvalho, G., Chirico, M., de Caceres, M., Durand, S., Evangelista, H., FitzJohn, R., Friendly, M., Furneaux, B., Hannigan, G., Hill, M., Lahti, L., McGlinn, D., Ouellette, M., Ribeiro Cunha, E., Smith, T., Stier, A., ter Braak, C., Weedon, J., 2022. Vegan: Community Ecology Package_. R package version 2.6-2, <https://CRAN.R-project.org/package=vegan>.

Paradis, E., 2010. Pegas: An R package for population genetics with an integrated-modular approach. Bioinformatics 26, 419–420. https://doi.org/10.1093/bioinformatics/btp696

Paradis, E., Schliep, K., 2019. Ape 5.0: An environment for modern phylogenetics and evolutionary analyses in R. Bioinformatics 35, 526–528. https://doi.org/10.1093/bioinformatics/bty633

Parker, I.M., Rodriguez, J., Loik, M.E., 2003. An evolutionary approach to understanding the biology of invasions: Local adaptation and general-purpose genotypes in the weed Verbascum ilose . Conservation Biology 17, 59–72. https://doi.org/10.1046/j.1523-1739.2003.02019.x

Pawlowski, J., Lecroq, B., 2010. Short rDNA barcodes for species identification in foraminifera. Journal of Eukaryotic Microbiology 57, 197–205. https://doi.org/10.1111/j.1550-7408.2009.00468.x

Pillet, L., Fontaine, D., Pawlowski, J., 2012. Intra-genomic ribosomal RNA polymorphism and morphological variation in elphidium macellum suggests inter-specific hybridization in foraminifera. PloS One 7. https://doi.org/10.1371/journal.pone.0032373

Prazeres, M., Morard, R., Roberts, T.E., Doo, S.S., Jompa, J., Schmidt, C., Stuhr, M., Renema, W., Kucera, M., 2020. High dispersal capacity and biogeographic breaks shape the genetic diversity of a globally distributed reef-dwelling calcifier. Ecol Evol 10, 5976–5989. https://doi.org/10.1002/ece3.6335

Quinn, T.P., Unwin, M.J., Kinnison, M.T., 2000. Evolution of temporal isolation in the wild: Genetic divergence in timing of migration and breeding by introduced chinook salmon populations. Evolution (N Y) 54, 1372–1385. https://doi.org/10.1111/j.0014-3820.2000.tb00569.x

R Core Team, 2022. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

Reiss, Z., Hottinger, L., 1984. The Gulf of Aqaba. Ecological Micropaleontology., Ecological. Ed. Springer-Verlag, New York. https://doi.org/10.1007/978-3-642-69787-6

Rilov, G., Galil, B., 2009. Marine Bioinvasions in the Mediterranean Sea – History, Distribution and Ecology 549–575. https://doi.org/10.1007/978-3-540-79236-9_31

Sakai, A.K., Allendorf, F.W., Holt, J.S., Lodge, M., Molofsky, J., With, K.A., Cabin, R.J., Cohen, J.E., Norman, C., Mccauley, D.E., Neil, P.O., Parker, M., Thompson, J.N., Weller, S.G., 2001. The population biology of invasive species. Annu. Rev. Ecol. Syst. 32, 305–32. https://doi.org/https://doi.org/10.1146/annurev.ecolsys.32.081501.114037

Schmidt, C., Morard, R., Prazeres, M., Barak, H., Kucera, M., 2016. Retention of high thermal tolerance in the invasive foraminifera Amphistegina lobifera from the Eastern Mediterranean and the Gulf of Aqaba. Mar Biol 163. https://doi.org/10.1007/s00227-016-2998-4

Shaltout, M., Omstedt, A., 2014. Recent sea surface temperature trends and future scenarios for the Mediterranean Sea. Oceanologia 56, 411–443. https://doi.org/10.5697/oc.56-3.411

Simberloff, D., Martin, J.L., Genovesi, P., Maris, V., Wardle, D.A., Aronson, J., Courchamp, F., Galil, B., García-Berthou, E., Pascal, M., Pyšek, P., Sousa, R., Tabacchi, E., Vilà, M., 2013. Impacts of biological invasions: What's what and the way forward. Trends Ecol Evol 28, 58–66. https://doi.org/10.1016/j.tree.2012.07.013

Smith, J.M., Haigh, J., 1974. The hitch-hiking effect of a favourable gene. Genet Res (Camb) 23, 23–35. https://doi.org/10.1017/S0016672308009579

Stuhr, M., Meyer, A., Reymond, C.E., Narayan, G.R., Rieder, V., Rahnenführer, J., Kucera, M., Westphal, H., Muhando, C.A., Hallock, P., 2018. Variable thermal stress tolerance of the reef-associated symbiont-bearing foraminifera Amphistegina linked to differences in symbiont type. Coral Reefs 37, 811–824. https://doi.org/10.1007/s00338-018-1707-9

Tajima, F., 1989. Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. Genetics 123, 585–595. https://doi.org/10.1093/genetics/123.3.585

Titelboim, D., Almogi-Labin, A., Herut, B., Kucera, M., Asckenazi-Polivoda, S., Abramovich, S., 2019. Thermal tolerance and range expansion of invasive foraminifera under climate changes. Sci Rep 9, 1–5. https://doi.org/10.1038/s41598-019-40944-5

Tsuchiya, M., Tazume, M., Kitazato, H., 2008. Molecular characterization of the non-costate morphotypes of buliminid foraminifers based on internal transcribed region of ribosomal DNA (ITS rDNA) sequence data. Mar Micropaleontol 69, 212–224. https://doi.org/10.1016/j.marmicro.2008.07.008

Tucker, A.E., Ackerman, M.S., Eads, B.D., Xu, S., Lynch, M., 2013. Population-genomic insights into the evolutionary origin and fate of obligately asexual Daphnia pulex. Proc Natl Acad Sci U S A 110, 15740–15745. https://doi.org/10.1073/pnas.1313388110

Wachowiak, W., Salmela, M.J., Ennos, R.A., Iason, G., Cavers, S., 2011. High genetic diversity at the extreme range edge: Nucleotide variation at nuclear loci in Scots pine (Pinus sylvestris L.) in Scotland. Heredity (Edinb) 106, 775–787. https://doi.org/10.1038/hdy.2010.118

Ward, S.M., Gaskin, J.F., Wilson, L.M., 2008. Ecological Genetics of Plant Invasion: What Do We Know? Invasive Plant Sci Manag 1, 98–109. https://doi.org/10.1614/ipsm-07-022.1

Weber, A.A.T., Pawlowski, J., 2014. Wide Occurrence of SSU rDNA Intragenomic Polymorphism in Foraminifera and its Implications for Molecular Species Identification. Protist 165, 645–661. https://doi.org/10.1016/j.protis.2014.07.006

Weiner, A.K.M., Morard, R., Weinkauf, M.F.G., Darling, K.F., André, A., Quillévéré, F., Ujiie, Y., Douady, C.J., de Vargas, C., Kucera, M., 2016. Methodology for single-cell genetic analysis of planktonic foraminifera for studies of protist diversity and evolution. Front Mar Sci 3, 1–15. https://doi.org/10.3389/fmars.2016.00255

Weinig, C., 2000. Plasticity Versus Canalization : Population Differences in the Timing of Shade-Avoidance Responses Author (s): Cynthia Weinig Published by : Society for the Study of Evolution Stable URL : https://www.jstor.org/stable/2640851 Society for the Study of Ev. Evolution (N Y) 54, 441–451. https://doi.org/10.1111/j.0014-3820.2000.tb00047.x

Weir, W., Capewell, P., Foth, B., Clucas, C., Pountain, A., Steketee, P., Veitch, N., Koffi, M., de Meeûs, T., Kaboré, J., Camara, M., Cooper, A., Tait, A., Jamonneau, V., Bucheton, B.,

Berriman, M., Macleod, A., 2016. Population genomics reveals the origin and asexual evolution of human infective trypanosomes. Elife 5, 1–14. https://doi.org/10.7554/eLife.11473

Xue, D.X., Graves, J., Carranza, A., Sylantyev, S., Snigirov, S., Zhang, T., Liu, J.X., 2018. Successful worldwide invasion of the veined rapa whelk, Rapana venosa, despite a dramatic genetic bottleneck. Biol Invasions 20, 3297–3314. https://doi.org/10.1007/s10530-018-1774-4

Yokes, M.B., Meric, E., Avsar, N., 2007. On the Presence of alien foraminifera Amphistegina lobifera Larsen on the coasts of the Maltese Islands. Aquat Invasions 2, 439–441. https://doi.org/10.3391/ai.2007.2.4.15

You, W., Yu, D., Liu, C., Xie, D., Xiong, W., 2013. Clonal integration facilitates invasiveness of the alien aquatic plant Myriophyllum aquaticum L. under heterogeneous water availability. Hydrobiologia 718, 27–39. https://doi.org/10.1007/s10750-013-1596-4

Yue, G.H., Li, J., Bai, Z., Wang, C.M., Feng, F., 2010. Genetic diversity and population structure of the invasive alien red swamp crayfish. Biol Invasions 12, 2697–2706. https://doi.org/10.1007/s10530-009-9675-1

Zenetos, A., Gofas, S., Morri, C., Rosso, A., Violanti, D., Garcia Raso, J., Cinar, M., Almogi-Labin, A., Ates, A., Azzurro, E., Ballesteros, E., Bianchi, C., Bilecenoglu, M., Gambi, M., Giangrande, A., Gravili, C., Hyams-Kaphzan, O., Karachle, P., Katsan, M., 2012. Alien species in the Mediterranean Sea by 2012. A contribution to the application of European Union's Marine Strategy Framework Directive (MSFD). Part 2. Introduction trends and pathways. Mediterr Mar Sci 13, 328–352. https://doi.org/https://doi.org/10.12681/mms.327

Zepeda-Paulo, F., Dion, E., Lavandero, B., Mahéo, F., Outreman, Y., Simon, J.C., Figueroa, C.C., 2016. Signatures of genetic bottleneck and differentiation after the introduction of an exotic parasitoid for classical biological control. Biol Invasions 18, 565–581. https://doi.org/10.1007/s10530-015-1029-6

Chapter 2

Flexible symbiosis in a larger benthic foraminifera along its invasion gradient in the Mediterranean Sea

This work is in preparation for submission.

Débora S. Raposo*[1], Christiane Hassenrück[1,2], Danna Titelboim[3], Sigal Abramovich[4], Antonio Caruso[5], Michal Kucera[1], Raphaël Morard[1]

[1] MARUM, Center for Marine Environmental Sciences, Universität Bremen, Bremen, Germany

[2] Leibniz Institute for Baltic Sea Research Warnemünde (IOW), Rostock-Warnemünde, Germany

[3] Department of Earth Sciences, University of Oxford, Oxford, United Kingdom

[4] Department of Earth and Environmental Sciences, Ben Gurion University of the Negev, Beer Sheva, Israel

[5] Dipartimento di Scienze della Terra e del Mare, Università degli studi di Palermo, Palermo, Italy

Data availability: All sequences generated in this study will be publicly available at ENA and the associated metadata will be available on the public repository PANGAEA Data Publisher once the paper is published. The code to the bioinformatics pipeline can be accessed in the repository https://github.com/Debora-raposo/Bioinformatics_pipeline_invasive_Amphistegina_eDNA.

ABSTRACT

The opening of the Suez Canal in 1869 triggered a dramatic migration of tropical species from the Red Sea into the Mediterranean Sea. Some invaders became highly abundant in their new locations, such as the benthic foraminifera *Amphistegina lobifera*. This prolific calcifier that relies on photosymbiosis with diatoms has recently expanded its invasion front to Sicily, where it copes with particularly low temperatures in winter. To understand the potential role of symbiosis in this invasion success we investigated the bacterial and eukaryotic microbiome composition of the holobiont with metabarcoding (16S and 18S rRNA gene). Specifically, we investigated if the *A. lobifera* recruits new symbiotic partners from the surrounding environment or if it modifies its symbiont composition from an internal pool during the invasion. We observed that the foraminifera microbiome, both bacterial and eukaryotic, was significantly less diverse and distinct in its composition compared to the ambient environmental DNA signal. The bacterial associations varied at the individual level consistent with a dynamic and temporary bacterial surface settlement, which may be actively counteracted by the host. Conversely, the eukaryotic microbiome was highly specific, dominated by diatoms from the araphid-pennate family. Different host populations along the invasion gradient showed associations with different diatom sequence variants, although most of these sequence variants were not detected in the environment. This suggests that either the foraminifera acquire their symbionts rarely or from an unknown source or that they keep an ancestral stock of symbionts, which are passed vertically by asexual reproduction, with new strains evolving within the host across the invasive range. Irrespective of the mechanism, our results indicate that the composition of the algal symbiosis is flexible, and its adjustment may allow the holobiont to face the novel thermal regime of the invaded environment.

Keywords: photosymbiosis, microbiome, environmental DNA, amplicon sequencing, Lessepsian invasion

1. INTRODUCTION

Ongoing global warming stimulates biological invasions of many species to higher latitudes and/or altitudes (Hickling et al., 2006; Walther et al., 2002). However, when the invasive species enter a new habitat, they encounter environmental conditions (climatic, physical, chemical, or biotic) that exceed those of their original location and induce physiological stress. Such new conditions can induce changes in microbiome associations in symbiont-bearing

organisms (e.g., Howells et al., 2016). These adaptations of symbiotic partnership can occur via microbiome frequency shifts (symbiont shuffling), acquisition of novel and beneficial microbiome strains (symbiont switching), or horizontal gene transfer between species (Rosenberg and Zilber-Rosenberg, 2011; Webster and Reusch, 2017). Alternatively, the invaders could already host symbiosis that possesses key adaptations (Joy, 2013; Schmidt et al., 2016a) that allows niche expansion of the holobiont (i.e., host and associated microbiome). With climate change, one cannot predict what will be the adaptive potential of invaders.

A perfect natural laboratory to study the invasion of marine species Is the Mediterranean Sea, where we can precisely trace the progress of the invasion. The Mediterranean Sea has been facing a dramatic biological invasion of tropical species from the Red Sea, known as the Lessepsian migration, since the last c.a. 150 years. The invasion was triggered by the opening of the Suez Canal, which created a mostly one-way migration corridor for Indo-Pacific species into the Mediterranean Sea. More than 680 invasive species (e.g., fish, crustaceans, mollusks, and other marine animals and plants) have been reported so far (Galil and Goren, 2014), causing a rapid change in the composition of the marine biota in the Mediterranean Sea (Rilov and Galil, 2009). The continued global warming in the Levantine basin proceeds at a pace faster than 0.1 °C/year (Ozer et al., 2017), enabling the tropical species to survive and further spread in the Mediterranean. However, although climate change results in higher summer temperatures, it does not affect the strong seasonality in the Mediterranean Sea which ranges from 11°C in winter to 30°C in summer (Borzelli and Ligi, 1999; Schmidt et al., 2016a). Therefore, as the invasion keeps progressing north and westwards in the Mediterranean, the invasive species encounter a thermal range that exceeds that of their native habitat, where seasonality is minimal (22°C in winter to 28°C in summer) (Schmidt et al., 2016a).

A particularly successful Lessepsian invader is the diatom-bearing larger benthic foraminifera (LBF) species, *Amphistegina lobifera*. This species inhabits shallow coastal waters, often adhering to macroalgae, pebbles, or coral reef fragments (Langer and Hottinger, 2000). Adult specimens can be dispersed by currents (Alve, 1999), and the passive mobility of the propagules or juveniles is possibly even greater (Alve and Goldstein, 2003), resulting in a broad species range (Prazeres et al., 2020). Its pioneer-invader populations were first observed in the Levantine basin in the 1960s (Langer, 2008), and after a few decades of progressive north and westward expansion (el Kateb et al., 2018; Langer and Mouanga, 2016; Meriç et al., 2008; Triantaphyllou et al., 2012; Yokes et al., 2007), they recently reached Sicily where they were

first observed in 2016 (Guastella et al., 2019). There, the invaders cope with winter temperature below 15 °C (Sorgente et al., 2003), compared to winter temperature in the Red Sea (native habitat) that remain above 22°C (Schmidt et al., 2016a). Yet, *A. lobifera* became extremely abundant, sometimes exceeding 50% of the benthic foraminifera assemblages in Sicily where the invasion front is located (Raposo et al., submitted).

Amphistegina lobifera has a well-established and documented association with pennate diatoms (Lee, 2006; Prazeres et al., 2021) that enhance its growth and calcification (Lee and Anderson, 1991). Under heat stress, *A. lobifera* expels its symbionts (Schmidt et al., 2016b) in a process analogous to reef-building corals that leads to the bleaching of the host (Hallock et al., 2006b; Schmidt et al., 2011). Moreover, initial studies on *Amphistegina* microbial communities have shown a shift in bacterial community composition with disturbance caused by heat stress (Prazeres, 2018; Prazeres et al., 2017). In other marine holobionts such as corals, an enhanced physiological resilience to environmental stress was related to either associations with more thermally tolerant endosymbiotic algae (zooxanthellae) genotypes (e.g., Grégoire et al., 2017; Jones and Berkelmans, 2010) or to beneficial bacterial consortia (Gilbert et al., 2012; Peixoto et al., 2021; Rosado et al., 2019; Zhang et al., 2021; Ziegler et al., 2017). Therefore, the ability to switch/shuffle symbiosis to better adapted strains is a great advantage to endure stressful environmental conditions.

In foraminifera, however, little is known about the host's flexibility to make new associations according to the taxa available in the surrounding environment. Like in many other symbiont-bearing organisms, the horizontal acquisition of symbionts is associated with sexual reproduction in Amphistegina (Dettmering et al., 1998; Harney et al., 1998). However, the main transfer of symbionts is thought to happen vertically during asexual reproduction (Dettmering et al., 1998; Harney et al., 1998). Therefore, to predict future range expansions of *A. lobifera* in the Mediterranean, it is important to understand their symbiosis associations across the invasive range, if they possibly acquire them from the surrounding environment and the role of their symbionts in the invasion success in colder temperatures.

Recent findings revealed no evidence of genetic differentiation between the invasive population of *A. lobifera* in the Mediterranean and the native population from the Red Sea (Prazeres et al., 2020, Raposo et al., submitted), precluding a potential adaptation of the host since the onset of the invasion that started 150 years ago. Therefore, we hypothesize that the adaptive success of the holobiont may derive from the flexibility in the symbionts. To investigate this, we addressed three main hypotheses: 1) the foraminifera acquire new thermal-

tolerant symbionts from the surrounding environment in the invaded habitat or over the course of the migration (symbiont switching); 2) the foraminifera keep the same symbiosis as in the native population composed by a pool of symbionts that are finely tuned for its physiological need, or 3) the foraminifera shift the proportion of the internal pool of symbionts (symbiont shuffling). For this, we investigated the composition and diversity of the bacterial and eukaryotic microbiome in four *A. lobifera* populations as well as in their surrounding environment (seawater and sediment) across the invasive range (native location in the Red Sea, pioneer invaded location in the Eastern Mediterranean Sea, and invasion front in Sicily)

2. MATERIALS AND METHODS

2.1 Sampling

To constrain the eukaryotic and bacterial microbiome of *A. lobifera* and its relatedness to the surrounding environment along its invasion range, we conducted two sampling campaigns to collect specimens from the source population (Red Sea), the early invaders (Eastern Mediterranean Sea) and the invasion front (Sicily; Figure 1). We conducted the first campaign in Sicily in September 2019 and the second in October 2019 in Israel to sample populations exposed to similar water temperatures (25-26 °C). In Sicily, we selected two locations to sample: Capo Passero in the southernmost invasive area (identified by (Guastella et al., 2019)) and Plemmirio in the northernmost invasive area (identified by Raposo et al. submitted). In Israel, we sampled at Shikmona, Haifa (Mediterranean Sea), and at Eilat, Gulf of Aqaba (Red Sea), where the presence of *A. lobifera* has already been primarily documented (Hottinger et al., 1993; Schmidt et al., 2016a; Titelboim et al., 2019).

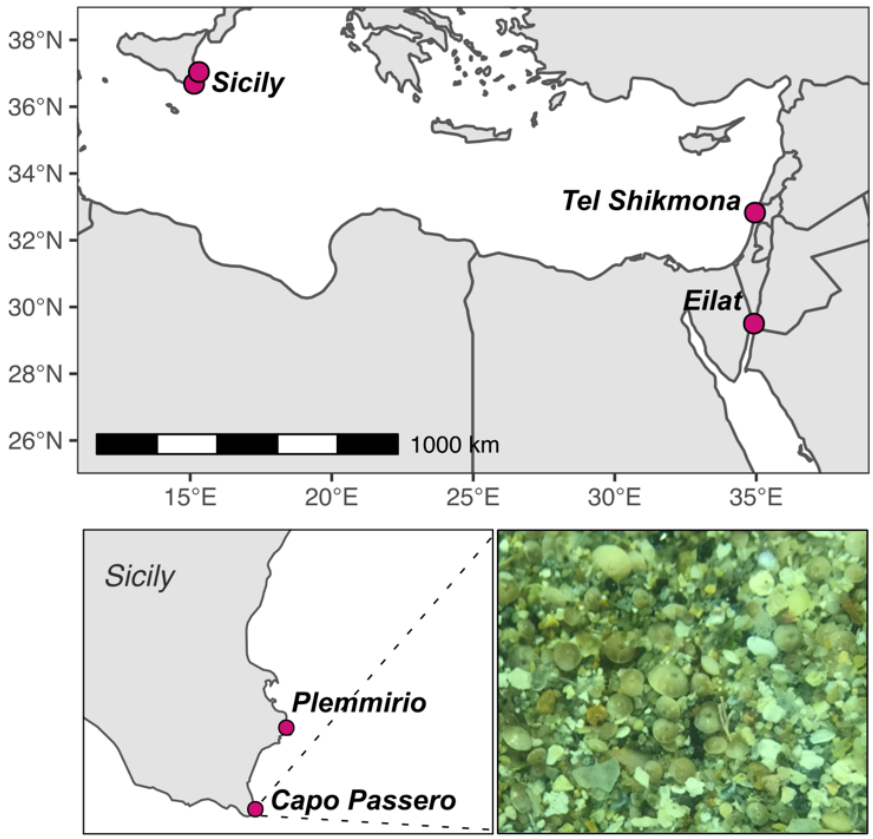


Figure 1. *Sampling sites in the invasion front in Sicily (Plemmirio and Capo Passero) and in Israel where the pioneer invaders (Tel Shikmona, Eastern Mediterranean) and the source population (Eilat, Red Sea) are located. The dominance of Amphistegina lobifera in Sicily is shown in the binocular view under 80x magnification of a sample from Capo Passero.*

At each location, we snorkeled to sample living foraminifera and the environmental DNA of their surrounding environment at two different depths (0.5 to 5 m) and from two substrates (pebbles and macroalgae), with an exception at Eilat where there were no macroalgae (Table S1). We brushed the collected substrates on the shore to detach the foraminifera, sieved the material to obtain a 63-500 µm size fraction and transferred this size fraction containing living foraminifera to 0.5 L jars filled with ambient seawater until further processing. We then recovered the material below 63 µm for the environmental DNA analysis and considered these environmental samples as "Sediment". We transferred this fine fraction material into 50 mL falcon tubes, let the particle settle, removed the water, and distributed the material into 2 mL Eppendorf vials. Finally, we collected seawater to have a comparable depiction of the environmental DNA in the water column. We collected seawater samples of 2 L at the same depths as the foraminifera and at the surface.

Back at the laboratory, we filtered the water samples on a 0.2 µm mixed cellulose ester (ME 24) membrane (Whatman/Cytiva, Germany) and froze them at -20°C as well as the sediment samples. We examined the foraminifera samples under the stereomicroscope and selected living specimens of *A. lobifera* characterized by brownish coloration (methods are detailed in Hallock et al., 2006a; Schmidt et al., 2016a; Stuhr et al., 2018). Thirty to sixty specimens were picked for each possible condition (substrates and depths) and individually transferred to

micropaleontological slides, where they were air-dried and stored at -20°C. All collected samples were shipped on dry ice to our laboratory in Bremen, where they were stored at -80°C until further processing.

2.2 DNA extraction, amplification, and sequencing

We planned to characterize the eukaryotic and bacterial microbiome of eight specimens per condition sampled (112 specimens in total), and we processed nine to 25 specimens for each condition to compensate for low DNA yield during extraction. We isolated each specimen in 50 µl of DOC buffer (Weiner et al., 2016), broke the thick calcite shell with a sterilized crusher, and incubated the samples overnight at 60°C. Then, the samples were centrifuged at 18.000 rpm for 5 min, and 1:10 dilutions of the supernatant were used as the DNA templates for sequencing library preparation.

To investigate the sediment microbiome, we weighted an aliquot of 250 mg of each sediment sample and extracted DNA with the Dneasy® PowerSoil® kit (Qiagen, Germany), following the instructions provided. For the seawater microbiome, we extracted the material retained on the ME membrane filters with the PureLink™ Plant Total DNA purification kit (Invitrogen, Germany). For this, we cut ¼ of the filter with a sterilized scissor, and the smaller pieces were then used for the extraction following the manufacturer's protocol. The concentrations of the DNA extracts of the environmental samples were measured with a Quantus™ Fluorometer (Promega, Germany) and standardized to 2.5 ng/µl.

To characterize the eukaryotic and bacterial communities of each sample, we targeted the hypervariable V4 region of the 18S SSU rRNA gene with the primers V4F (5′-CCAGCASCYGCGGTAATTCC− 3′) and V4R (5′- ACTTTCGTTCTTGATYRA-3′) (Stoeck et al., 2010) and the V3-V4 region of the 16S SSU rRNA gene with the primers 341F (5′-CCTACGGGNGGCWGCAG−3′) and 805R (5′-GACTACHVGGGTATCTAATCC-3′) (Herlemann et al., 2011). For both amplifications, we used tagged primers with eight nucleotides appended to each primer's 5′-end to enable multiplexing of all PCR products in one sequencing library (Esling et al., 2015). The primer-tag combinations are provided in Table S2.

All diluted templates were amplified in two technical replicates using a specific tagged primer pair with the PHUSION® Hot-start II polymerase (Thermo Fisher Scientific, USA) in a PCR mix containing MilliQ water, 5x PHUSION Green HF Buffer (final concentration: 1×), each

primer (final concentration: 0.2 µmol/µl), DMSO (final concentration: 0.75 µmol/µl), $MgCl_2$ (final concentration: 2.4 µmol/µl), dNTP mix (final concentration: 0.2 µmol/µl) and Phusion Green Hot Start II polymerase (final concentration: 0.02 U/µl), and 5 µl of the DNA template within a final volume of 25 µl. The thermal cycling was as follows: initial denaturation at 98°C for 2 min followed by 35 cycles of 10 s of denaturation at 98°C, annealing for 30 s at 52°C for the eukaryotes or 60°C for the bacteria, and extension at 72°C for 30 s, followed by a final extension at 72°C for 2 min for the single-cell samples. The same conditions were applied for the amplification of the environmental samples but reducing to the number of PCR cycles to 32. At least one negative control of extraction (NC) was included in every PCR batch to monitor any contamination during DNA extraction and amplification. The obtained PCR products were visualized by a 1.5% agarose gel electrophoresis stained with ethidium bromide. The samples in which the two technical replicates were successful were selected as well as the NCs of each PCR batch. All products were purified using the QIAquick PCR purification kit (Qiagen, Germany) following the manufacturer's instructions and the DNA concentration was measured with a Quantus™ Fluorometer (Promega, Germany).

Eight library pools were generated for each marker by equimolar pooling of 37 to 41 purified PCR products. The environmental samples and the single-cell foraminifera samples were placed in different pools, and samples having the same tagged pairs were sequenced in different pools. To acquire additional sequences from the environmental samples, they were pooled with twice as much DNA as the single-cell foraminifera samples, therefore they were not as heavily multiplexed. Pools were sequenced on the Illumina MiSeq platform using the 2 × 300 bp run paired-end protocol yielding paired-end reads. Sequencing was conducted at the Center for Human Genetics and Genetic Counselling (ZHG) (University of Bremen, Germany).

2.3 Bioinformatics pipeline

The obtained fastQ files containing all amplicon sequences including the NCs were deposited to GFBio/ENA. Sequence reads were demultiplexed and the primer sequences were clipped with cutadapt (Martin, 2011). We only assigned sequences to a sample where both the R1 and R2 indices were correctly identified. The remaining steps were conducted in the software R 4.1.1 (R Core Team, 2022), using the DADA2 package and following the protocol for Illumina amplicon data analysis (Callahan et al., 2016). Briefly, the pipeline consisted of trimming and filtering the reads to remove low-quality sequences , denoising R1 and R2 reads independently

by sequencing library to correct sequencing errors, merging pair-end (R1 and R2) reads, and removing chimeric sequences and singletons. The remaining sequences were used to construct the amplicon sequence variant (ASV) table. The taxonomic classification followed the k-mer-based approach of RDP (Vinje et al., 2015) and was performed against the curated databases: PR^2 (del Campo et al., 2018; Guillou et al., 2013) for the eukaryotic community and 16S SILVA v138.1 database (Glöckner et al., 2017; Quast et al., 2013; Yilmaz et al., 2014) for the bacterial community. The number of sequences retained in each step of the pipeline is reported in Table S3.

The code to the bioinformatics pipeline of our datasets can be accessed in the repository https://github.com/Debora-raposo/Bioinformatics_pipeline_invasive_Amphistegina_eDNA.

The NCs were removed after we confirmed that they were either sufficiently different from the samples from the same PCR batch (> 90% Bray-Curtis dissimilarities), or they showed neglectable DNA concentration, or they did not show any bands in the PCR gels. The ASV profiles from the two technical replicates were merged in a final filtering step. For the eukaryotic dataset, we kept only the ASVs present in both technical replicates. Because the taxonomic composition of the bacterial community between technical replicates of the same specimen was more dissimilar (Figure S1), we used relaxed criteria to not discard genuine signal from the analyses. As a result, we chose to keep the ASVs that were either present in both technical replicates or, if present only in one technical replicate, occurred in both technical replicates of at least one other specimen. In both cases, when merging the technical replicates, the number of sequences was summed for each ASV that was kept. The rarefaction curves were checked for saturation to ensure that all sample types had been sequenced sufficiently deep (Figure S2).

Since we focused on the symbiotic interactions in our analyses, we removed the ASVs that belonged to land plants, metazoans, and other larger taxa, as well as taxa that could be substrate (e.g., the red algae *Jania rubens*) from the eukaryotic dataset. To focus on the bacterial composition, we removed chloroplast and mitochondrial sequences, as well as ASVs annotated as eukaryotic or archaeal. To reduce ambiguity in the interpretation of the results, we removed the unclassified taxa in the larger taxonomic categories: Phylum (Division for the eukaryotic dataset), and Class. Because the taxonomy of the ASVs is not yet resolved in the 18S SILVA v138.1 and PR^2 databases for all observed genera or species, our analyses were based on the ASV level.

2.4 Data analyses

To constrain whether the foraminifera could possibly acquire symbionts from the environment, we compared the bacterial and eukaryotic microbiome composition between the single-cell foraminifera, sediment, and seawater samples. For this, we performed non-metric multidimensional scaling (NMDS) based on the Bray-Curtis dissimilarities of ASV proportions (Figure 3). We plotted inverse Simpson indexes to investigate how diversity variated for each sample type and population (Figure 3). To compare the degree of overlap between the different microbiome communities, we conducted analyses of similarities (ANOSIM) grouping by sample type. To observe how many ASVs the foraminifera had in common with the environment, we performed UpSet plots comparing the intersection of the bacterial ASVs in the different sample types (Figure 4) and constraining each population individually (Figure S5). Similarly, we did the same approach for the eukaryotic microbiome and for the diatoms' ASVs only (Figure 4), which constituted the vast majority (99.4%) of the number of reads (Figure 2; Figure S6).

Finally, to see the degree of relatedness of the microbiome composition within the different foraminifera populations across the invasive range, we focused the following analyses on single-cell foraminifera samples only. We conducted NMDS analyses for their bacterial microbiome and their diatom symbionts, and we plotted the distance to centroid of beta dispersion analyses to investigate the multivariate homogeneity of variances within populations (Figure 5). To test the effect of population, depth, and substrate type (foraminifera collected either from pebbles or macroalgae) in the differences in microbiome composition we then performed a permutational multivariate analysis of variance (PERMANOVA) based on Bray-Curtis dissimilarities. Post-hoc PERMANOVA for all possible pairwise comparisons within a significant factor was conducted and the p-value was adjusted for false discovery rate (FDR) in multiple comparisons. We calculated the PERMANOVA and the post-hoc pairwise comparisons with the adonis2 function in the *vegan* R package (Oksanen et al., 2022). The ASVs counts were converted to relative proportions prior to all analyses based on Bray-Curtis dissimilarities (NMDS, ANOSIM, and PERMANOVA).

2.5 Phylogeographic analyses of diatom symbionts

To further explore the community structure of the diatom microbiome across the invasive range, we explored their phylogenetic relationship because the vast majority of the eukaryotic

symbionts belonged to this group. To this end, we used only diatoms sequences, automatically aligned them with MAFFT (Katoh and Standley, 2013) and inferred a phylogenetic tree using RaxML-NG (Kozlov et al., 2019) with 100 non-parametric bootstraps and using the substitution model TVM+I+G that was selected with Modeltest-NG (Darriba et al., 2020; Flouri et al., 2015).

The weighted Unifrac distances for all sample pairs were calculated with the *phyloseq* R package (McMurdie and Holmes, 2013). In addition, we tested the hypothesis of speciation by distance. Linear regressions were performed with Unifrac distance as a function of pairwise geographical distances for comparisons (a) with the source population and (b) for all foraminifera populations. The comparisons restricted to the source populations enable us to discover the impact of migration on their diatom communities, whereas the comparisons for all populations allow us to examine the effect of intrapopulation and interpopulation variability in this process.

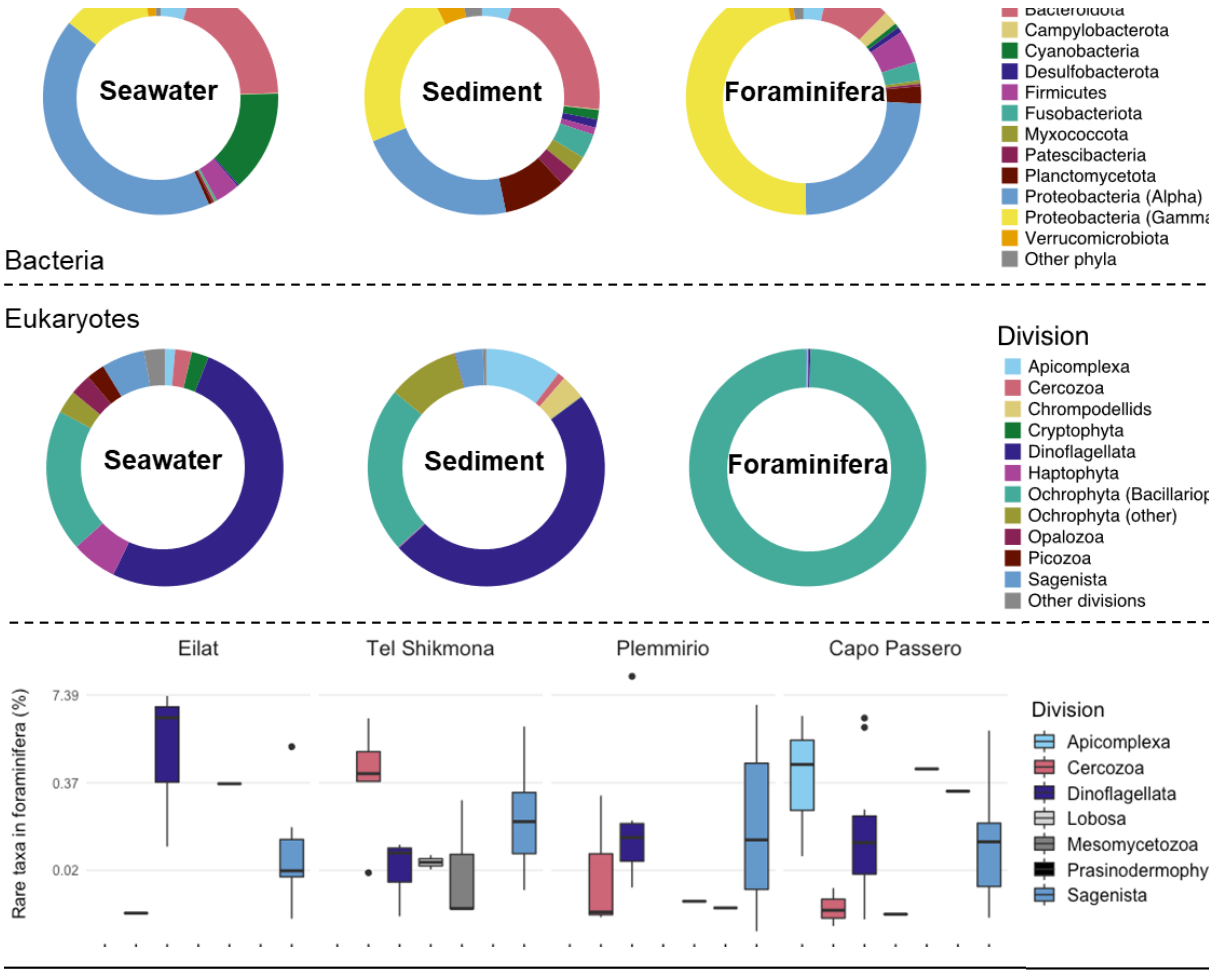
Next, to observe the degree of mutations between the diatom symbionts in the different populations, we constructed a haplotype network with the *pegas* R package (Paradis, 2010), building a minimum spanning network using Bandelt et al.'s algorithm (Bandelt et al., 1999). Since haplotype networks are designed for closely related species, we selected only the 60 most dominant ASVs which belonged to the Araphid-pennate family (the family that dominated all foraminifera populations). The dataset was rarified to the minimum sequencing depth (1433 sequences per sample) prior to this analysis to improve visualization and to avoid misinterpretation due to differences in sequencing effort.

3. RESULTS

3.1 Bacterial and eukaryotic community diversity

We successfully extracted, amplified, and sequenced 112 single-cell foraminifera, 32 filters, and 32 sediment samples for bacterial and eukaryotic analyses (Table S1). We obtained 14,447,166 raw reads for the prokaryotic (bacteria and archaea) data set and 11,837,770 raw reads for the eukaryotic data set. After processing the samples through the cleaning and filtering steps in the bioinformatics pipeline (Table S3), 2,927,842 reads were retained for the bacterial data set and 9,613,242 reads for the eukaryotic data set.

A total of 5,649 ASVs were obtained in the dataset for the bacterial community from the foraminifera, 11,750 ASVs from the sediment, and 9,863 ASVs from the seawater microbiome. The classes Alphaproteobacteria and Gammaproteobacteria dominated both the foraminiferal and environmental microbiomes (Figure 2A), with the foraminifera populations in the invasion



ifferent sample types (seawater, sediment, and foraminifera). Colors in donut plots represen ifferent phyla (or divisions for eukaryotes). Taxa with proportions below 1% were condense "Other phyla/divisions". Box plots show average percentage of rare taxa (not from Ochrop ivision) in the foraminifera samples (C). The y axis was log-transformed to allow visualizatic axa at lower proportion. The taxonomic compositions of the bacterial and the eukar icrobiomes within each population are reported in the supplementary material (Fig S3 and Fig espectively).

front in Sicily displaying a higher dominance of Gammaproteobacteria (Figure S3) compared to the pioneer population in Tel Shikmona and the source population in Eilat. The class

Bacteroidia also dominated the environmental samples, mostly in the invasive populations, while the class Cyanobacteria predominantly dominated in Eilat (Figure S3).

For the eukaryotic dataset, we retained 1005 ASVs from the foraminifera, 1539 ASVs from the sediment, and 3085 ASVs from the seawater microbiome, after removing 5338 ASVs attributed to land plants, metazoans and other larger taxa, and taxa that could be a substrate. The sediment and seawater revealed a much more diverse eukaryotic microbiome than the foraminifera (Figure 2B). The ASV-based rarefaction curves reached saturation for the eurayotic data set (Figure S2). Although eight divisions were identified in the foraminifera microbiome (Apicomplexa, Cercozoa, Dinoflagellata, Lobosa, Mesomycetozoa, Ochrophyta, Prasinodermophyta, and Sagenista), the Ochrophyta division constituted 99.4% of the total number of sequences. Within Ochrophyta, 99.7% of the ASVs belonged to the Class Bacillariophyta (diatoms), and within Class Bacillariophyta 98.8% of the ASVs belonged to the Araphid-pennate family, revealing a highly selected eukaryotic microbiome. This extreme selection is sustained in the within-population analyses (Figure S4). The remaining divisions occurred rarely and not consistently for every individual within the populations sampled (Figure 2C).

Table 1. ANOSIM and Post-hoc test based on Bray-Curtis dissimilarities of bacterial and eukaryotic microbiomes, grouping by sample type

Data set	Grouping by	R	Significance	Group 1	Group 2	R	P adj (FDR)
Bacteria	Sample type	0.882	0.001	foraminifera	sea water	0.982	0.001
				foraminifera	sediment	0.787	0.001
				sediment	sea water	0.996	0.001
Eukaryotes	Sample type	0.866	0.001	foraminifera	sea water	0.966	0.001
				foraminifera	sediment	0.891	0.001
				sediment	sea water	0.816	0.001

3.2 Foraminifera vs environmental microbiome

The NMDS reflected the stark difference between the composition of the sample types for both bacterial and eukaryotic datasets (Figure 3A), which was confirmed by the significant p values ($p < 0.001$) in the ANOSIM (Table 1). The strongest differentiation was between the

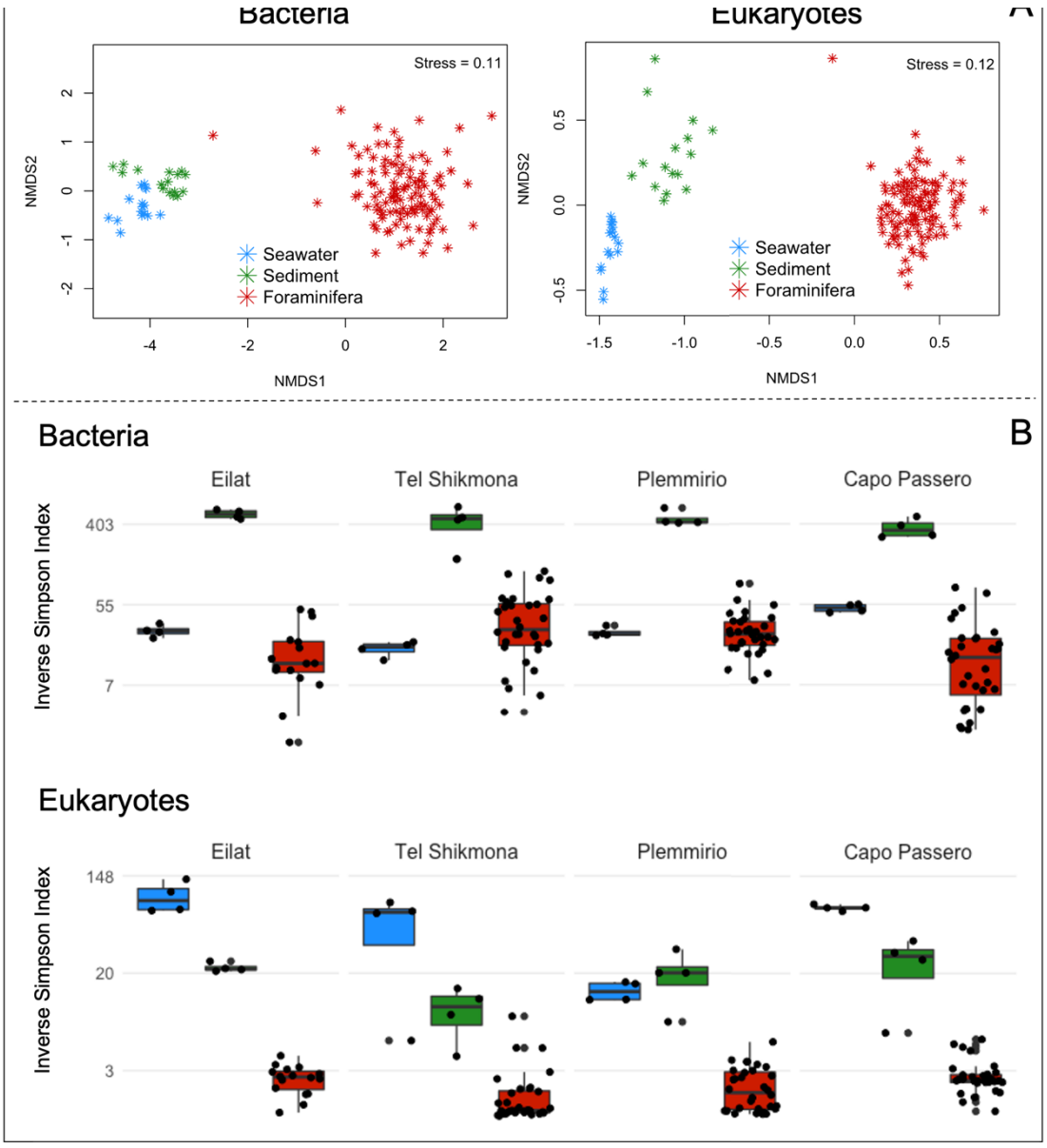
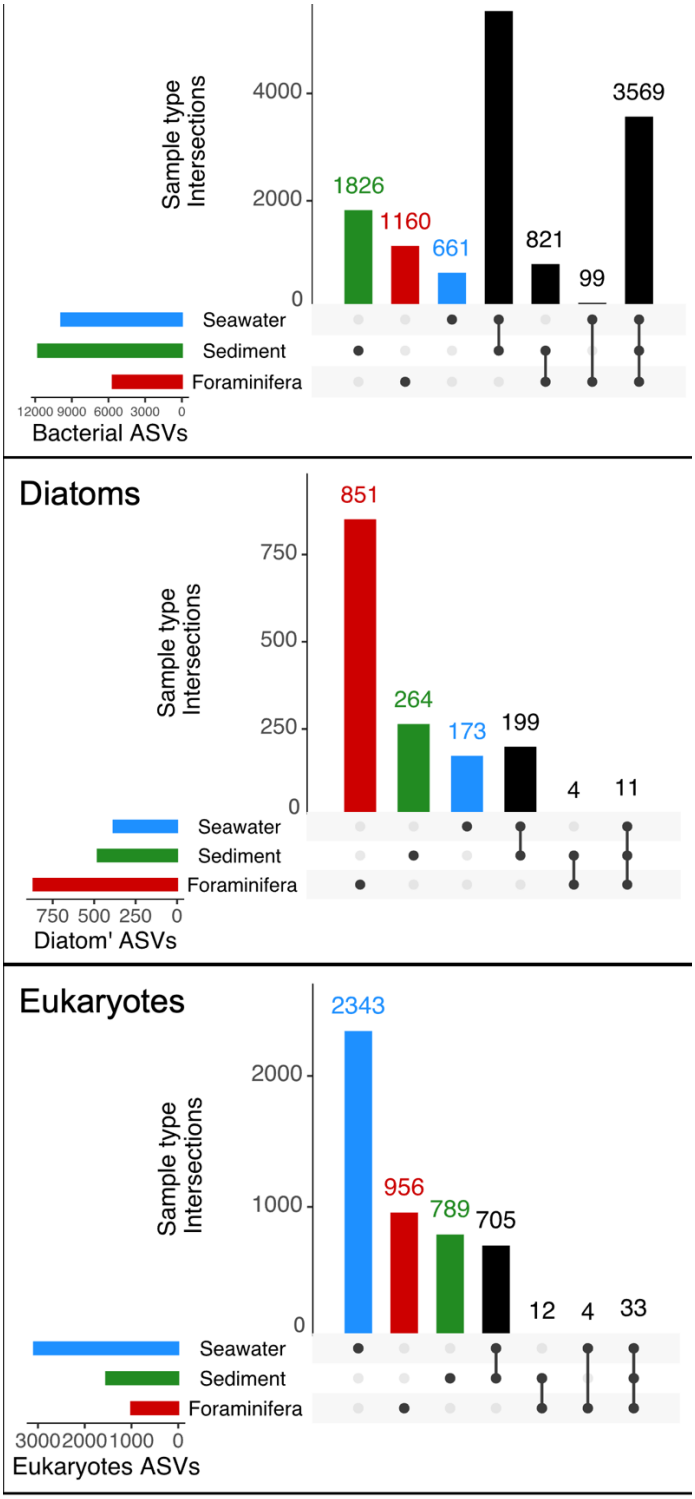


Figure 3. *NMDS of Bray-Curtis dissimilarities (A) and inverse Simpson index (B) of the bacterial and eukaryotic microbiome from the different sample types (foraminifera, seawater, and sediment). Populations ordered from source in Eilat, to pioneer invaders in Tel Shikmona, and invasion front in Plemmirio and Capo Passero*

foraminifera and their surrounding environment, and the water and sediment samples showed a stronger separation in the eukaryotic than in the bacterial data set. The inverse Simpson index indicated that also at the ASV level the foraminifera had a much less diverse eukaryotic microbiome than their surrounding environment. For the bacterial community the pattern is different; while both foraminiferal and seawater microbiome were much less diverse than the sediment microbiome, the foraminifera revealed alpha diversity that is comparable to, and occasionally higher than, the surrounding seawater samples (Figure 3B).

We observed 3317 bacterial ASVs that occur in all sample types (24 % of total ASVs), while only 33 common ASVs were observed for the eukaryotic dataset (0.6% of the total eukaryote ASVs; Figure 4). The difference is kept when narrowing the analysis to diatoms, which constitute most of the foraminifera eukaryotic microbiome. Only 11 diatom's ASVs were common to all sample types (0.7% of diatoms' ASV diversity), and the foraminifera harbored a total of 866 diatoms ASVs, which is higher than those detected in the environmental samples (651 ASVs in total). These

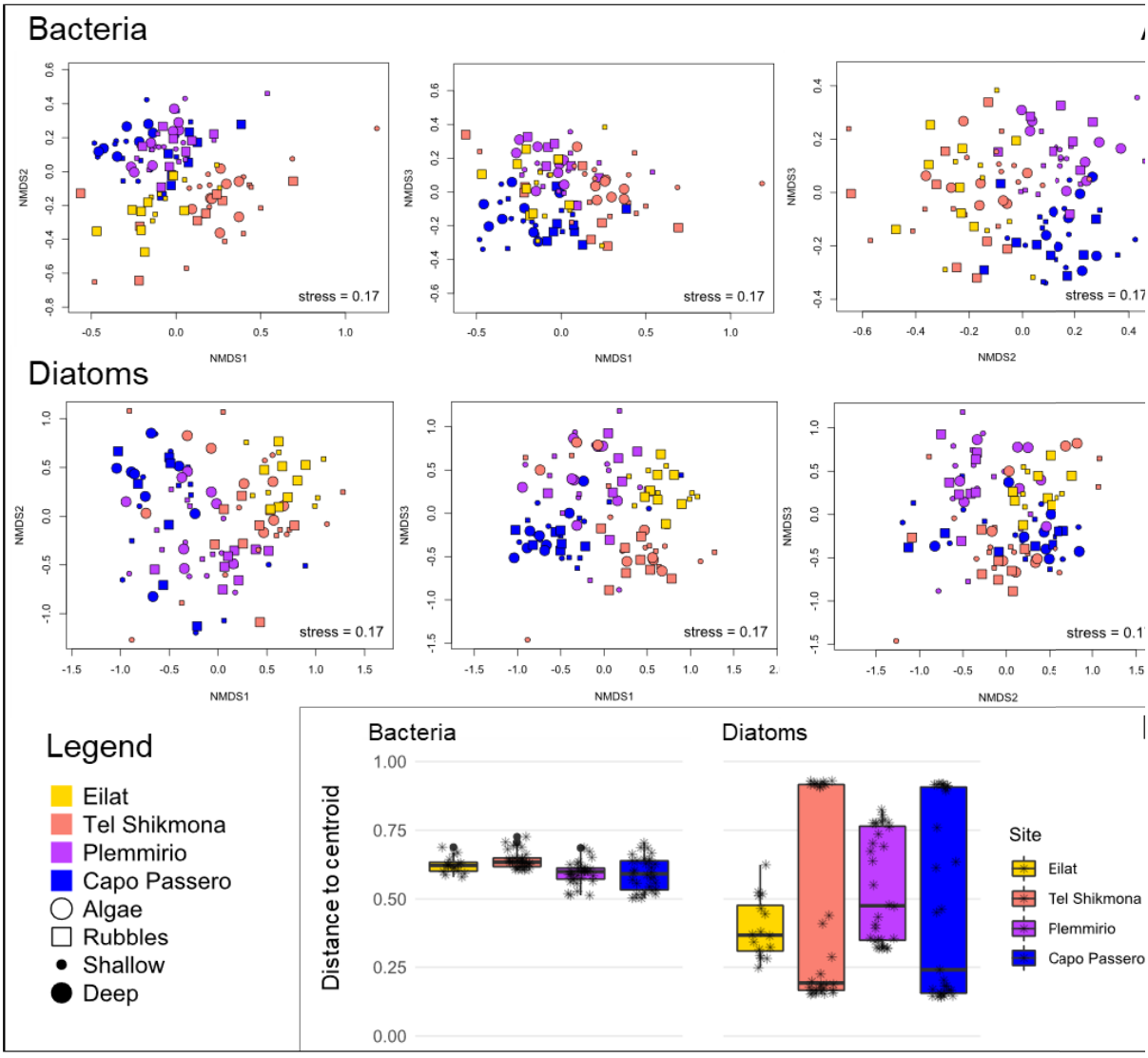


diatom, and all eukaryotic ASVs in the different sampl types (seawater, sediment and foraminifera)

results were also observed in the analyses within populations (Figure S5, Figure S6).

3.3 Foraminiferal microbiome across the invasive range

The NMDS showed that the foraminifera microbiome primarily clustered by population for both bacterial and diatom communities (Figure 5). Both datasets are highly structured with two



microbiome and diatoms (which covered most of the eukaryotic microbiome). Each data po represents a foraminifera individual. The NMDS for the entire eukaryotic microbiome is repor in Figure S7. Beta dispersion (distance to centroid) within populations is shown as boxplot a jitter for each foraminifera individual (B). Colors show the populations across the invasive ran

clusters: one for the Sicilian populations (invasion fronts, Plemmirio and Capo Passero) and the other for the Israeli populations (pioneer invaders and source population, Tel Shikmona

and Eilat, respectively). The PERMANOVA confirmed the difference per population (Tables 2 and 3) and revealed that depth and substrate type have a minor, although significant, effect on the bacterial composition but not on the diatom composition. The distance to centroids in the beta dispersion analyses revealed a consistently high level of heterogeneity of the bacterial microbiome within foraminifera populations (distance to centroids always superior to 0.5). However, for the diatoms, we observe a bimodal distribution in all but the source population, characterized by mostly small dissimilarities among most samples, and a few larger differences cause by samples with strongly divergent community composition. Eilat and Tel Shikmona populations hosted the fewest and the highest number of bacterial and diatoms ASVs, respectively (Figures S5 and S6).

Table 2. PERMANOVA based on Bray-Curtis dissimilarities of foraminifera bacterial microbiome to test the effect of different populations, substrates, and depths (P-values based on 999 permutations). Post-hoc PERMANOVA pairwise comparisons (with FDR correction) tested for Population (Eil = Eilat, Shi = Tel Shikmona, Ple = Plemmirio, Cap = Capo Passero). Different groups in post-hoc are indicated by different letters.

Factor	Df	Sum Of Sqs	R2	F	P	Post-hoc pairwise comparison
Population	3	5.03	0.107	4.58	**0.001**	Eil-A, Shi-B, Ple-C, Cap-D
Depth	1	0.50	0.011	1.37	**0.014**	Shallow-A, Deep-B
Substrate	1	0.96	0.020	2.63	**0.001**	Macroalgae-A, Pebbles-B
Population:Depth	3	1.39	0.029	1.26	**0.002**	-
Population:Substrate	2	1.76	0.037	2.41	**0.001**	-
Depth:Substrate	1	0.56	0.012	1.53	**0.005**	-
Population:Depth:Substrate	2	1.04	0.022	1.42	**0.003**	-
Residual	98	35.83	0.761			
Total	111	46.78	1			

Table 3. PERMANOVA based on Bray-Curtis dissimilarities of foraminifera diatoms to test the effect of different populations, substrates, and depths (P-values based on 999 permutations). Post-hoc PERMANOVA pairwise comparisons (with FDR correction) tested for Population (Eil = Eilat, Shi = Tel Shikmona, Ple = Plemmirio, Cap = Capo Passero). Different groups in post-hoc are indicated by different letters.

	Df	Sum of Sqs	R2	F	P	Post-hoc pairwise comparison
Population	3	16.63	0.349	18.88	**0.001**	Eil-A, Shi-B, Ple-C, Cap-D
Substrate	1	0.19	0.004	0.66	0.771	
Depth	1	0.25	0.005	0.84	0.592	
Population:Substrate	2	0.45	0.009	0.76	0.779	
Population:Depth	3	0.64	0.014	0.73	0.877	
Substrate:Depth	1	0.40	0.008	1.35	0.163	
Population:Substrate:Depth	2	0.61	0.013	1.04	0.386	
Residual	97	28.47	0.598			
Total	110	47.64	1			

3.4 Phylogeographic analysis of symbiotic diatoms

The phylogeographic analysis to investigate the dissimilarity in symbiotic diatoms across the invasive range showed a positive relationship between Unifrac distance and geographic distance from the source population ($R2 = 0.764$, p-value < 0.001; Figure 6A). The model including all possible comparisons revealed a reduced R2 ($R2 = 0.362$, p-value < 0.001; Figure 6A). This is caused by a higher intrapopulation variability across the invasive range compared to the source population in Eilat.

The haplotype network consisted of 60 ASVs representing the most dominant diatoms (Figure 6B). The structure of the network showed a clear geographical separation. Distinct ASVs dominated each population, and there were only a few shared ASVs between populations with a pronounced degree of separation between the Sicilian and the Levatin populations. We observed only a few shared ASVs between all Mediterranean populations, few co-occurrences between Tel Shikmona and Eilat, and no shared ASVs between Eilat and Sicily. The further structure seems to occur within populations when ASVs with only one number of mutations from a particularly dominant ASV arise. This pattern is observed in all *A. lobifera* populations, although it was more pronounced in Sicily (Plemmirio).

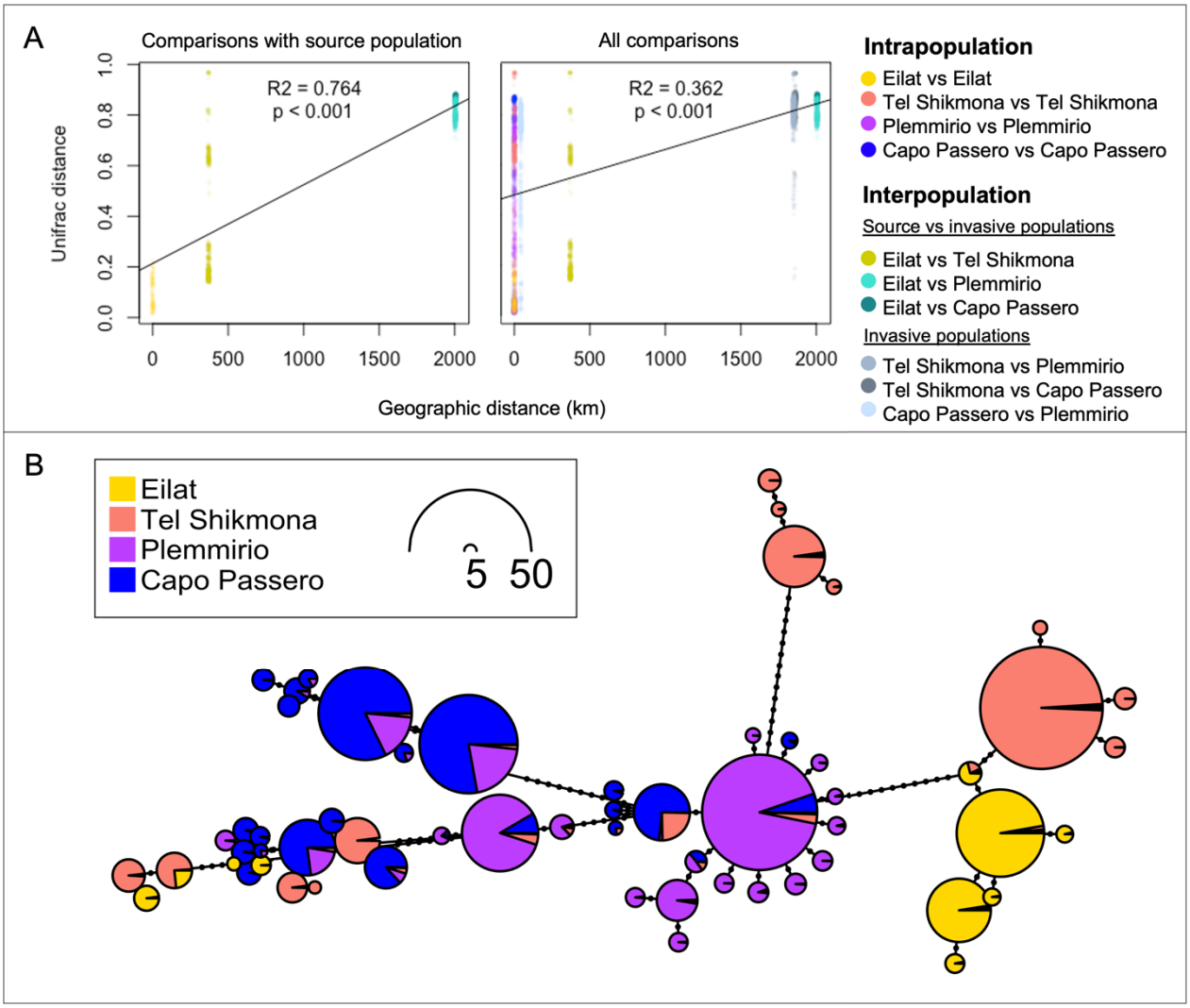


Figure 6. *Linear correlation between Unifrac distance of A. lobifera diatoms as a function of geographic distance: from the source populations (R-squared: 0.764; p < 0.001) and for all possible comparisons (R-squared: 0.362; p < 0.001) (A). Haplotype network based on the 60 most dominant diatom ASVs from the raphid-pennate family. Origin of an ASV is shown by color, dots in the connections represent the number of mutations, and the size of pie charts shows the square root of the number of sequences, rescaled to show a maximum of 50 sequences (B)*

4. DISCUSSION

Consistent with previous studies (Lee, 2006; Prazeres et al., 2021) we observed that the foraminifera eukaryotic microbiome was mostly composed of diatoms from the Araphid pennate family, and they are possibly the only mandatory symbionts in *A. lobifera*. Although other eukaryotes were present (i.e., Cercozoa, Dinoflagellata, Sagenista), they were rare (< 0.5 %) or absent in the foraminifera. It is likely that these rare taxa are epiphytes or transient food signals that were preserved in the cytoplasm of the host and appear in the sequences despite being functionally irrelevant to the symbiont pool (Prazeres et al., 2021). Indeed, it has been

shown that *A. lobifera* relies on heterotrophic feeding on algae and bacteria for nutrient supplementation (ter Kuile and Erez, 1988). Potential signs of an enhanced heterotrophic feeding are observed in the pioneer invasive population in the Eastern Mediterranean (Tel Shikmona), which hosted the highest number of both bacterial (Figure S5) and diatom (Figure S6) ASVs. Based on our field observations, the site in Tel Shikmona is marked by higher energy waves that bring unsteady and less clear water conditions compared to the original habitat in Eilat. In addition, the higher seasonality in Tel Shikmona with warmer summers and colder winters (Borzelli and Ligi, 1999; Schmidt et al., 2016a) exposes the population to a broader range of environmental conditions that is reflected in the more diverse associated microbiome.

The narrow selection observed in the eukaryotic microbiome is not observed in the bacterial microbiome. The bacterial microbiome was comparably much richer and more diverse and the majority of the bacterial ASVs appear at low relative abundance, with high variability at the individual foraminifera level. This is consistent with the rare biosphere concept (i.e., highly diverse pool of rare microbial species) which is applicable to most host-microbial associations in nature (Lynch and Neufeld, 2015) and has been also documented in *Amphistegina* from the Great Barrier Reef (Prazeres, 2018; Prazeres et al., 2017). Prazeres (2018) associated the high diversity of bacteria with a possible response to bleach stress (loss of their diatoms) and suggested that the bleached foraminifera could be relying on the ingestion of bacteria to meet their energy requirements. The high dissimilarity of the bacterial microbiome at the individual foraminifera level associated with the significant effect of depth and substrate type in their composition likely indicates bacterial consortia consistent with either a dynamic and temporary surface settlement in the foraminifera or heterotrophic feeding on bacteria. However, it is important to highlight that, both in the present study and in the study by Prazeres (2018), the statistical models were able to explain only up to 27% of the microbial variation across the different samples. This means that most of the microbial variation is explained by factors that we were not yet possible to constrain. This emphasizes the need for continuous investigations to determine the factors that promote the relationship between LBF and host-associated microbial communities.

Nevertheless, it seems that the foraminifera can regulate their bacterial microbiome given the dominance of Gammaproteobacteria, while in the environmental samples the Alphaproteobacteria is the most dominant class (Figure 2). This regulation of the bacterial community seems to be enhanced in the two populations in the invasion front, which show a

substantially more pronounced dominance of Gammaproteobacteria compared to the levant populations, despite no increase in dominance of this taxon in the environmental samples (Figure S3). This raises the hypothesis of a possible strategy of the foraminifera host to adapt to the new conditions by selecting certain bacteria. In reef-building corals, which have comparable ecological requirements as symbiont-bearing foraminifera (Hallock et al., 2003), the association with different bacterial consortia has been revealed as a crucial and successful solution to cope with environmental stress (Rosado et al., 2019; Zhang et al., 2021; Ziegler et al., 2017). However, the function(s) of the dominant bacterial taxa and their specific role in the foraminifera holobiont remains unknown and further investigations to confirm this hypothesis would be necessary.

Our previous research on the population structure of *A. lobifera* has shown that the invasion success in the Mediterranean Sea is not related to genetic differentiation of the host, i.e., the genotype of the invasive populations, including the invasion front in Sicily, is the same as the source population from the Red Sea (Raposo et al., submitted). In addition, the invasion of the new habitat seems to come at the cost of switching to exclusive asexual reproduction mode in the Mediterranean Sea (Raposo et al., submitted). Therefore, to explain the success of invasion despite the stressful environmental conditions, our first hypothesis was that a symbiont shifting with taxa available in the environment would be taking place in the invasive populations. In this scenario, the host with the ability to acquire new algal symbionts and bacterial microbiome, that increase its niche, would be able to thrive in the new location (Schmidt et al., 2018). Opposite to this hypothesis, our results indicate that the foraminifera populations do not seem to acquire their diatoms from their immediate surrounding environment, given that they had less than 1.3% of mutual ASVs (11 ASVs in common out of 866 ASVs, Figure 4) and were significantly different in the ANOSIM. Moreover, none of these ASVs in common were dominant in the foraminifera samples. This is consistent with a previous study on LBFs from Lee et al. (1992) which found that the diatoms that form symbioses are rare, or not detected at all, in the environment. One could argue that the absence of overlap between the symbiotic and the environmental diatoms in our results could be a bias of a lower sequencing depth in the environmental samples. However, this is unlikely since all samples showed well-saturated rarefaction curves (Figure S2). Opposed to the pattern of the diatom symbionts, the foraminifera bacterial microbiome was much more comparable to the surrounding environment, showing a higher number of mutual ASVs than exclusive ASVs between the foraminifera and the environmental samples (Figure 4). Therefore, it seems that the internal

pool of symbiont is tightly controlled by the host, and not only randomly selected from the environment.

Like in many other symbiont-bearing organisms, in *A. lobifera*, the horizontal acquisition of symbionts is associated with sexual reproduction. Because the tiny gametes (2-3 μm) cannot transport the symbionts, the zygotes must acquire them from the environment (Dettmering et al., 1998; Harney et al., 1998). Conversely, asexual reproduction is associated with the vertical transfer of symbionts to the offspring. Moreover, recent findings revealed that *A. lobifera* in the Mediterranean Sea are mainly reproducing asexually (Raposo et al., submitted). This would in principle accentuate even more vertical transmission of symbionts to the offspring and reduce horizontal acquisition from the environment. This supports our findings of reduced horizontal acquisition of diatoms in the invasive populations. Alternatively, nothing speaks against the acquisition of the symbionts in adult specimens and/or over the course of migration or within their surrounding environment but from a different season that we did not capture with our sampling strategy.

To test our second hypothesis that the invasive foraminifera would carry the same symbiosis as in the source population, we compared the foraminifera diatoms from the different populations. Intriguingly, even though all foraminifera consistently hosted diatoms from the same family (Araphid pennate), they did show flexible diatom symbioses at the ASV level across the different populations in the invasive range (Figure 5A, Table 3). Since the dissimilarities in diatom symbionts could not be explained by different associations with the surrounding environment, we investigated their phylogeographic relationships. The pairwise comparisons based on the Unifrac and geographical distances between the symbiotic diatoms (Figure 6A) revealed that the diatoms from the source population in Eilat have a lower intrapopulation variability compared to the ones from the invasive populations. This indicates a higher diatom diversity in the invasive population. Also, we observed that the phylogenetic distances are strongly related to the geographic distances between populations, where the symbiotic diatoms in the invasion front are the most genetically distant from the symbiotic diatoms in the source population. This result is supported by the haplotype network that shows several new ASVs appearing within each population closely related to a dominant ASV (Figure 6B). Because these new ASVs are mostly population-specific, we can presume they are responsible for the high dissimilarity between the symbiotic diatoms in the invasive range.

However, the question about the origin of these new diatoms ASVs persists. These results need an answer that explains both the occurrence of new strains of diatoms which are strongly

population-specific and the fact that these ASVs were likely not obtained from the immediate surrounding environment. One possibility to consider is that new strains of diatoms are evolving within the host. In this context, the foraminifera could be actively selecting their diatoms, and the diatoms could be evolving inside the foraminifera. Either way, the host could keep these new taxa within the population by transferring them to their offspring by vertical transmission during asexual reproduction (Raposo et al., submitted). This could represent an adaptation of the host to keep the most beneficial symbionts by shifting the proportion of the internal pool of symbionts, such as observed in reef-building corals (Cunning et al., 2015). This means that our third hypothesis of a symbiont shuffling is possibly taking place. In addition, we propose a fourth hypothesis of the evolution of new diatom strains inside the foraminifera host, regardless of the invasive context. This hypothesis could also explain the higher richness of symbiotic diatoms than the free-living environmental diatoms and the observed lack of horizontal transmission.

During horizontal transmission, it is expected that the host acquires and/or transmits its symbionts to the environment or to other hosts (Bright and Bulgheresi, 2010). However, because the symbiotic diatoms do not develop their frustules (siliceous cell envelopes) when living inside the foraminifera (Lee, 2006; Leutenegger, 1983), they are less prone to survive when transmitted horizontally. Therefore, they possibly have become specialized to live inside the host (e.g., host specialization, Lajoie and Parfrey, 2022). Such an example of symbiont evolution independently from free-living taxa (non-symbiont strains of the same taxonomic lineage) was already documented in bacterial symbionts from aphid hosts (Burke et al., 2009). Moreover, in marine holobionts such as corals and sponges, coevolution has been documented between the host and their bacterial symbionts (O'brien et al., 2019). However, the evolutionary mechanism that underpins the transition from a free-living to symbiotic life history remains largely unknown (González-Pech et al., 2019). In addition, our findings open questions about the advantage for the holobiont to have such a selected and specialized internal pool of diatoms. Would the foraminifera be "farming" their diatoms for heterotrophic feeding? Or would the host become an evolutionary "hub" for diatoms? Either way, regardless of the mechanism, the invasive *A. lobifera* found a way to dynamically adapt its symbiotic composition, which may be related to their ability to thrive under thermal stress in the new locations.

5. CONCLUSION

In this study, we used single-cell metabarcoding to investigate eukaryotic and bacterial associations of the foraminifer *A. lobifera* across its invasive range and its relationship with the microbiome of the surrounding environment. The bacterial associations varied at the individual level consistent with a dynamic and temporary bacterial surface settlement, which may be actively counteracted by the host. Conversely, the foraminifera host a highly selected eukaryotic microbiome almost exclusively composed of diatoms (Class Bacillariophyta) from the Araphid-pennate family. Different host populations along the invasion gradient showed associations with different diatom sequence variants, although these sequence variants were rare, or not detected, in the surrounding environment. This means that either new strains of diatoms are evolving within the host across the invasive range, which are passed vertically by asexual reproduction, or the foraminifera acquire their different symbionts over the course of migration or during a different season. Either way, the host seems to dynamically adapt its symbiotic composition in the invaded environment. These results advance our knowledge of the host-microbiome relationship during an invasion phenomenon and can improve comprehension of the importance of the microbiome to cope with thermal stress in the new habitat.

Acknowledgments

We thank Dr. Giulia Visconti, director of the Plemmirio marine reserve. Sampling was possible thanks to the agreement with the Dipartimento di Scienze della Terra e del Mare (DiSTeM) of Palermo University. We thank Israel Nature and Parks Authority for supporting both sampling campaigns conducted in Israel under permit 42055/2018 and Dr. Miguel Frada, who kindly provided lab access at the IUI biological station. This study was funded by the BMBF-MOST cooperation in Marine Sciences Grant No. 03F0820A “ForaInva”, and by the Cluster of Excellence “The Ocean Floor—Earth’s Uncharted Interface” funded by the German Research Foundation (DFG).

Supplementary material

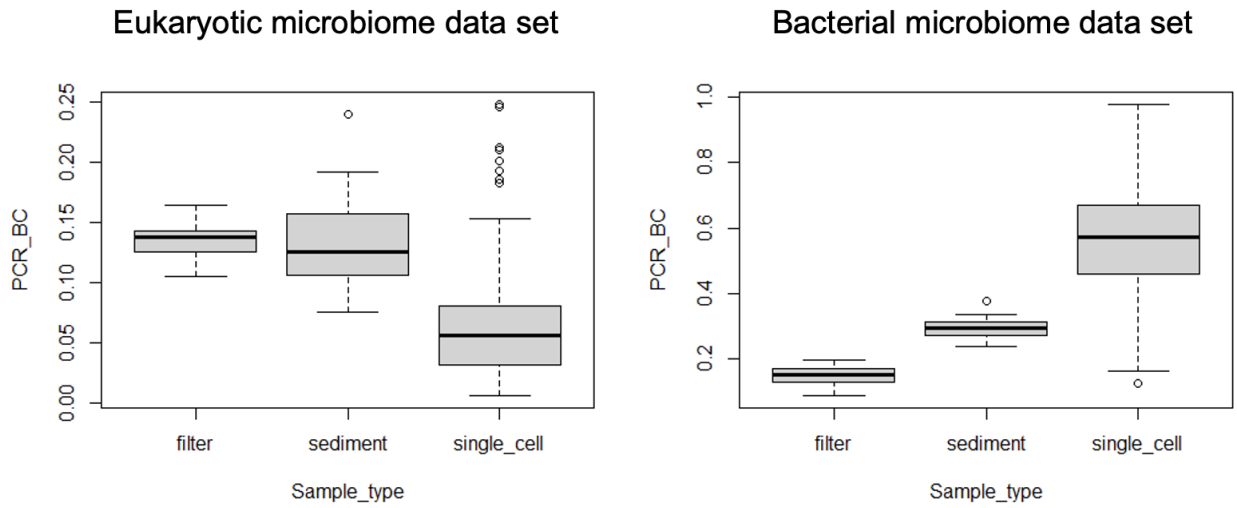


Figure S1. *Boxplot of Bray-Curtis' (BC) Dissimilarities between PCR/technical replicates (y) per sample type (x) for both eukaryotic and bacterial datasets.*

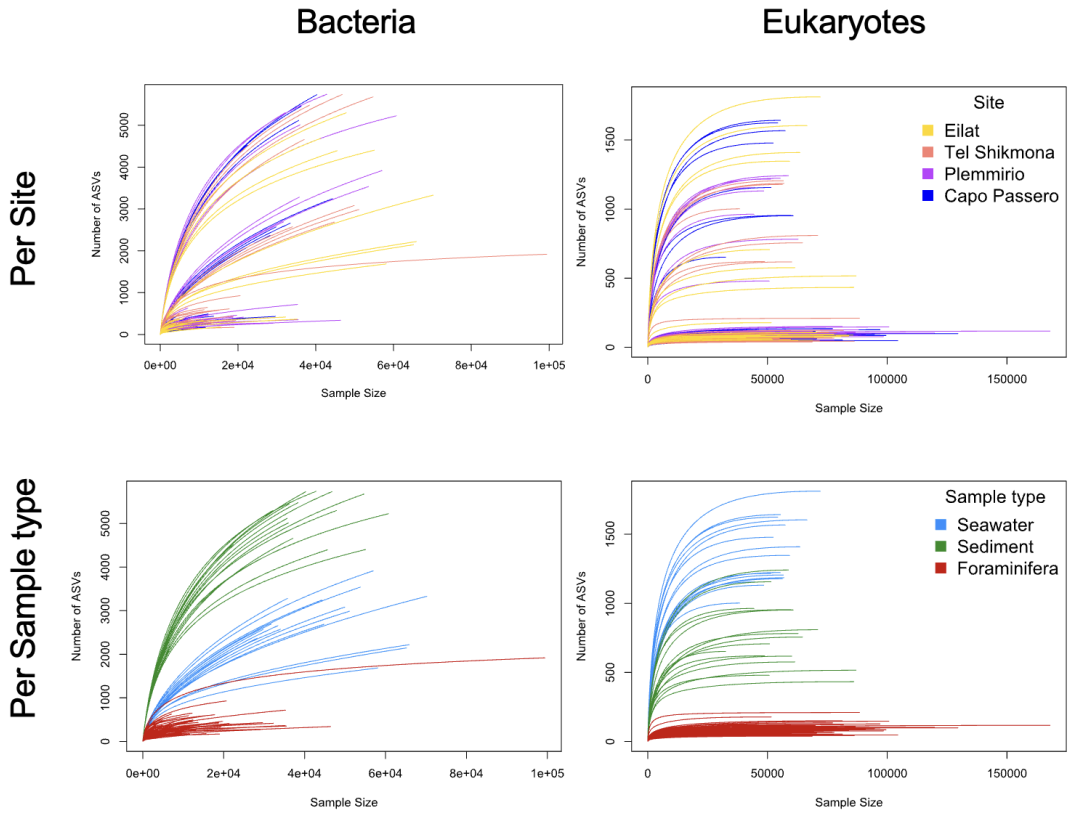


Figure S2. *Rarefaction curves for bacterial and eukaryotic microbiomes of single-cell foraminifera, seawater and sediment samples.*

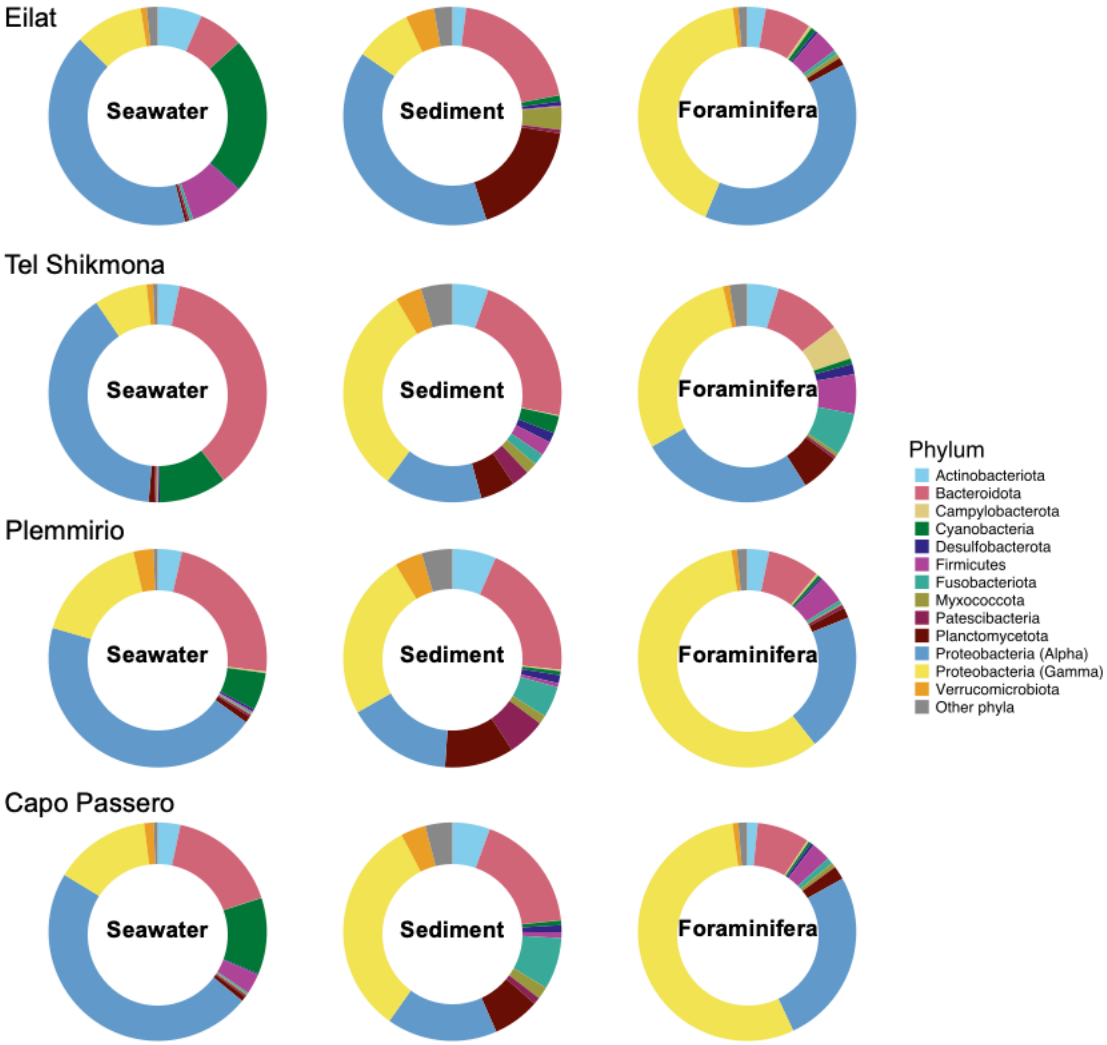


Figure S3. *Bacterial community compositions in foraminifera and environmental samples within each site*

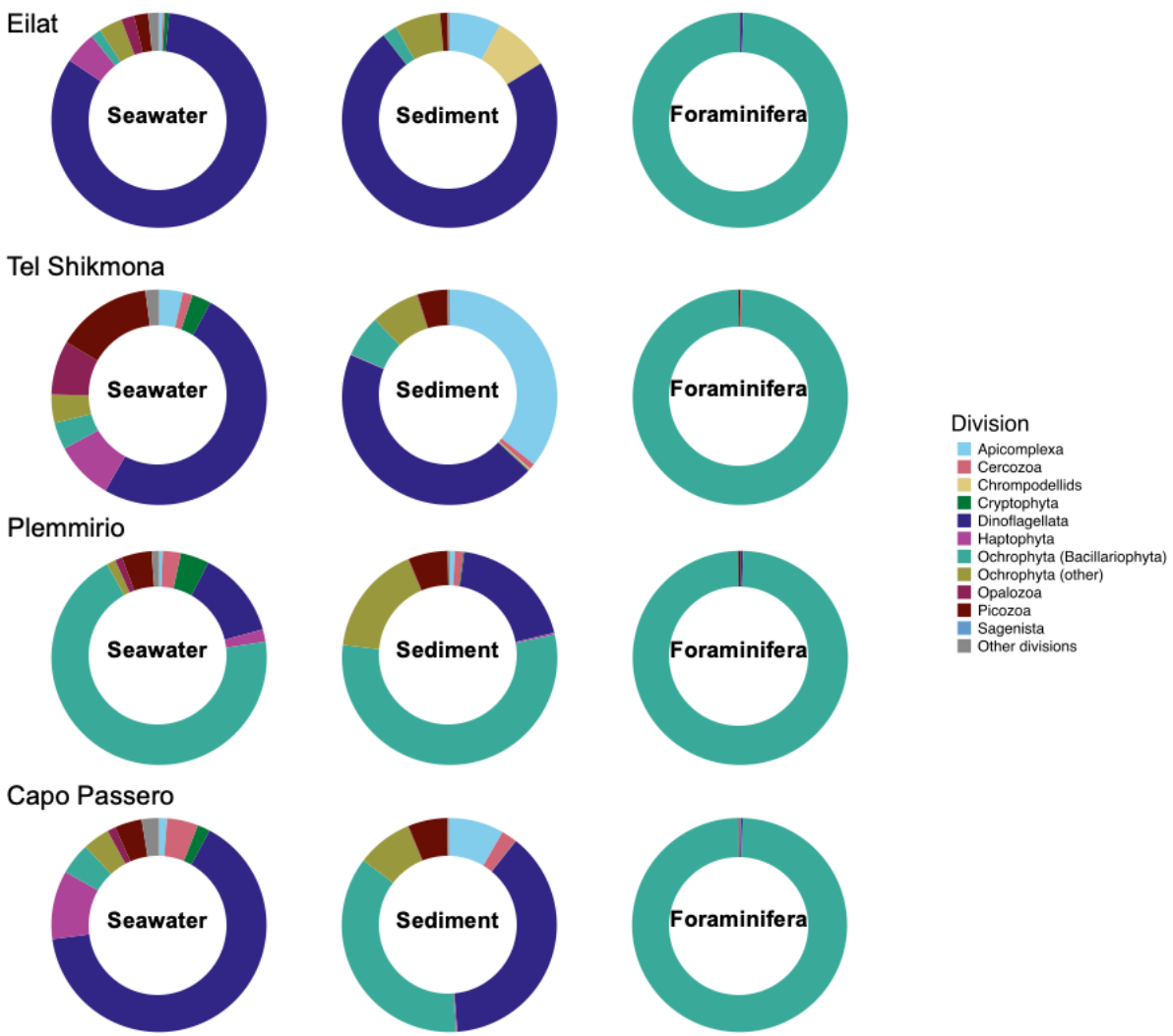


Figure S4. *Eukaryotic community composition in foraminifera and environmental samples within each site*

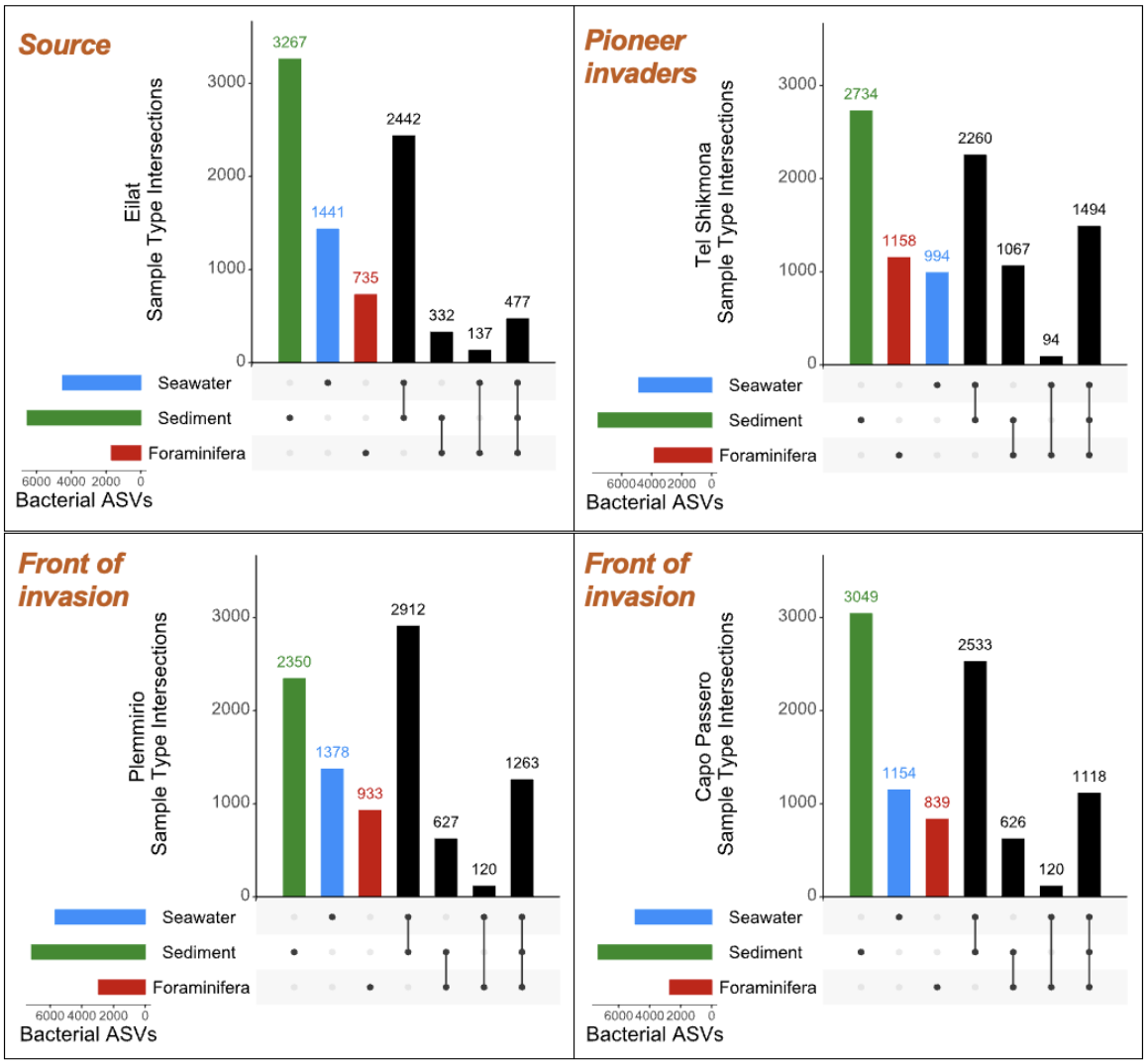


Figure S5. *Foraminifera bacterial ASVs intersection between sample types, grouping by population.*

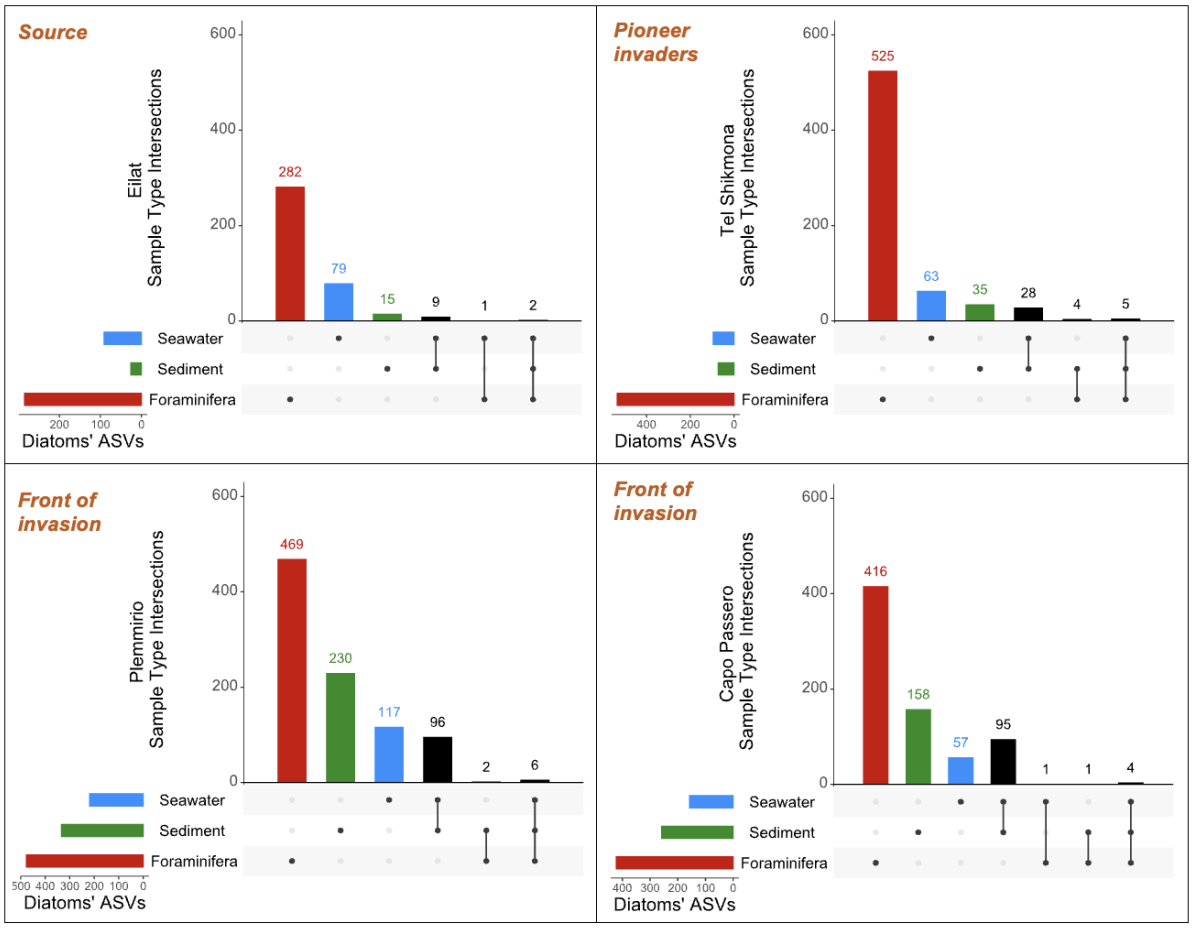


Figure S6. *Foraminifera diatoms' ASVs intersection between sample types, grouping by population.*

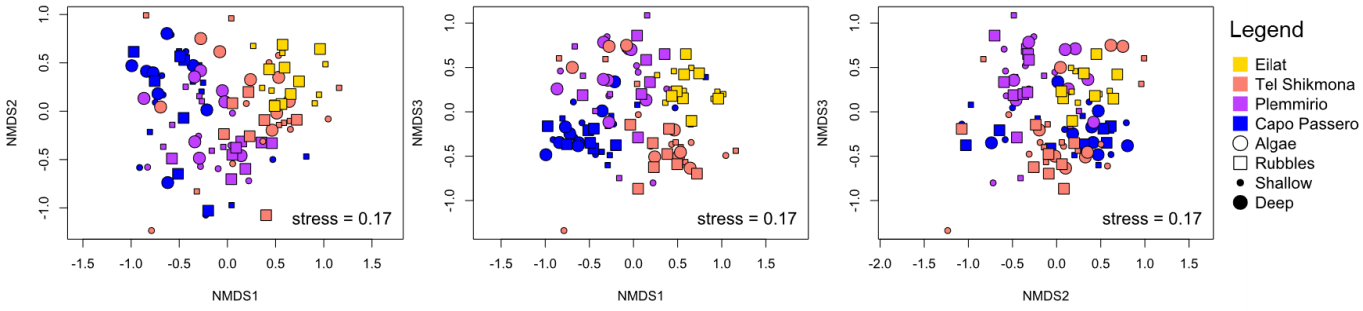


Figure S7. *NMDS based on Bray-Curtis dissimilarities between foraminifera eukaryotic microbiome (populations as colors).*

Table S1 Sampling sites and number of samples in each different condition: depth, substrate type, water column height (in case of seawater samples).

Condition	Foraminifera	Sediment	Seawater
Capo Passero			
2 m, 36.686483 N 15.137911 E			
algae	8	1	
rubbles	8	1	
bottom			1
surface			1
4 m, 36.686500 N 15.138139 E			
algae	8	1	
rubbles	8	1	
bottom			1
surface			1
Plemmirio			
2 m, 37.039667 N 15.306472 E			
algae	8	1	
rubbles	8	1	
bottom			1
surface			1
4 m, 37.039672 N 15.306688 E			
algae	8	1	
rubbles	8	1	
bottom			1
surface			1
Tel Shikmona			
0.5 m, 32.825671 N 34.955123 E			
algae	8	1	
rubbles	8	1	
bottom			1
surface			1
1.5 m, 32.825778 N 34.955028 E			
algae	8	1	
rubbles	8	1	
bottom			1
surface			1
Eilat			
1 m, 29.502028 N 34.917722 E			
rubbles	8	2	
bottom			1
surface			1
5 m, 29.501963 N 34.917832 E			
rubbles	8	2	
bottom			1
surface			1

Table S2 Primers sequences list

Primer_Prok	Primer_sequence	Tag	Primer_sequence_end
341F_A	AGAGCTAGCCTACGGGNGGCWGCAG	AGAGCTAG	CCTACGGGNGGCWGCAG
341F_B	ATACTCTCCCTACGGGNGGCWGCAG	ATACTCTC	CCTACGGGNGGCWGCAG
341F_C	ATGTCGTCCCTACGGGNGGCWGCAG	ATGTCGTC	CCTACGGGNGGCWGCAG
341F_D	GAGTCGAGCCTACGGGNGGCWGCAG	GAGTCGAG	CCTACGGGNGGCWGCAG
341F_E	GATCTCGCCCTACGGGNGGCWGCAG	GATCTCGC	CCTACGGGNGGCWGCAG
341F_F	GATGTGTGCCTACGGGNGGCWGCAG	GATGTGTG	CCTACGGGNGGCWGCAG
341F_G	GTAGTGACCCTACGGGNGGCWGCAG	GTAGTGAC	CCTACGGGNGGCWGCAG
341F_H	GTATCTGCCCTACGGGNGGCWGCAG	GTATCTGC	CCTACGGGNGGCWGCAG
341F_I	GTGCTCAGCCTACGGGNGGCWGCAG	GTGCTCAG	CCTACGGGNGGCWGCAG
341F_J	TAGCTGTCCCTACGGGNGGCWGCAG	TAGCTGTC	CCTACGGGNGGCWGCAG
341F_K	TATGCTGCCCTACGGGNGGCWGCAG	TATGCTGC	CCTACGGGNGGCWGCAG
341F_L	TGATCGTGCCTACGGGNGGCWGCAG	TGATCGTG	CCTACGGGNGGCWGCAG
341F_M	TGTCTGAGCCTACGGGNGGCWGCAG	TGTCTGAG	CCTACGGGNGGCWGCAG
341F_N	TGTGTCTCCCTACGGGNGGCWGCAG	TGTGTCTC	CCTACGGGNGGCWGCAG
805R_A	ACACAGCAGACTACHVGGGTATCTAATCC	ACACAGCA	CTACHVGGGTATCTAATCC
805R_B	ACTCTACGGACTACHVGGGTATCTAATCC	ACTCTACG	CTACHVGGGTATCTAATCC
805R_C	AGATCGCGGACTACHVGGGTATCTAATCC	AGATCGCG	CTACHVGGGTATCTAATCC
805R_D	CGACTACAGACTACHVGGGTATCTAATCC	CGACTACA	CTACHVGGGTATCTAATCC
805R_E	CGTCAGCGGACTACHVGGGTATCTAATCC	CGTCAGCG	CTACHVGGGTATCTAATCC
805R_F	GTACTGCGGACTACHVGGGTATCTAATCC	GTACTGCG	CTACHVGGGTATCTAATCC
805R_G	GTATCACAGACTACHVGGGTATCTAATCC	GTATCACA	CTACHVGGGTATCTAATCC

Primer_Euk	Primer_sequence	Tag	Primer_sequence_end
V4F_A	ACACACACCCAGCASCYGCGGTAATTCC	ACACACAC	CCAGCASCYGCGGTAATTCC
V4F_B	ACGACTCTCCAGCASCYGCGGTAATTCC	ACGACTCT	CCAGCASCYGCGGTAATTCC
V4F_C	ACGCTAGTCCAGCASCYGCGGTAATTCC	ACGCTAGT	CCAGCASCYGCGGTAATTCC
V4F_D	ACTATCATCCAGCASCYGCGGTAATTCC	ACTATCAT	CCAGCASCYGCGGTAATTCC
V4F_E	ACTGCTGACCAGCASCYGCGGTAATTCC	ACTGCTGA	CCAGCASCYGCGGTAATTCC
V4F_F	AGACATCTCCAGCASCYGCGGTAATTCC	AGACATCT	CCAGCASCYGCGGTAATTCC
V4F_G	AGTCTACACCAGCASCYGCGGTAATTCC	AGTCTACA	CCAGCASCYGCGGTAATTCC
V4F_H	CAGATCACCCAGCASCYGCGGTAATTCC	CAGATCAC	CCAGCASCYGCGGTAATTCC
V4F_I	CATACTGCCCAGCASCYGCGGTAATTCC	CATACTGC	CCAGCASCYGCGGTAATTCC
V4F_J	CATATACTCCAGCASCYGCGGTAATTCC	CATATACT	CCAGCASCYGCGGTAATTCC
V4F_K	CATCATATCCAGCASCYGCGGTAATTCC	CATCATAT	CCAGCASCYGCGGTAATTCC
V4F_L	CGACTCATCCAGCASCYGCGGTAATTCC	CGACTCAT	CCAGCASCYGCGGTAATTCC
V4R_A	CAGAGACGACTTTCGTTCTTGATYRA	CAGAGACG	ACTTTCGTTCTTGATYRA
V4R_B	CAGATGACACTTTCGTTCTTGATYRA	CAGATGAC	ACTTTCGTTCTTGATYRA
V4R_C	CAGTATGCACTTTCGTTCTTGATYRA	CAGTATGC	ACTTTCGTTCTTGATYRA
V4R_D	CATAGTATACTTTCGTTCTTGATYRA	CATAGTAT	ACTTTCGTTCTTGATYRA
V4R_E	CATGTGCTACTTTCGTTCTTGATYRA	CATGTGCT	ACTTTCGTTCTTGATYRA
V4R_F	CGAGAGACACTTTCGTTCTTGATYRA	CGAGAGAC	ACTTTCGTTCTTGATYRA
V4R_G	CGAGTACGACTTTCGTTCTTGATYRA	CGAGTACG	ACTTTCGTTCTTGATYRA
V4R_H	CGATGTAGACTTTCGTTCTTGATYRA	CGATGTAG	ACTTTCGTTCTTGATYRA
V4R_I	CGTATAGCACTTTCGTTCTTGATYRA	CGTATAGC	ACTTTCGTTCTTGATYRA
V4R_J	CGTGATGTACTTTCGTTCTTGATYRA	CGTGATGT	ACTTTCGTTCTTGATYRA
V4R_K	GAGATAGTACTTTCGTTCTTGATYRA	GAGATAGT	ACTTTCGTTCTTGATYRA
V4R_L	GAGTGTCTACTTTCGTTCTTGATYRA	GAGTGTCT	ACTTTCGTTCTTGATYRA

Table S3 Number of sequences retained for each step in the bioinformatics pipeline for both prokaryotic and eukaryotic datasets.

(Only available in the digital version of this dissertation)

References

Alve, E., 1999. Colonization of new habitats by benthic foraminifera: A review. Earth Science Reviews 46, 167–185. https://doi.org/10.1016/S0012-8252(99)00016-1

Alve, E., Goldstein, S.T., 2003. Propagule transport as a key method of dispersal in benthic foraminifera (Protista). Limnol Oceanogr 48, 2163–2170. https://doi.org/10.4319/lo.2003.48.6.2163

Bandelt, H.J., Forster, P., Röhl, A., 1999. Median-joining networks for inferring intraspecific phylogenies. Mol Biol Evol 16, 37–48. https://doi.org/10.1093/oxfordjournals.molbev.a026036

Borzelli, G., Ligi, R., 1999. Autocorrelation Scales of the SST Distribution and Water Masses Stratification in the Channel of Sicily. Journal of Atmospheric and Oceanic Technology 16, 776–781. https://doi.org/https://doi.org/10.1175/1520-0426(1999)016<0776:ASOTSD>2.0.CO;2

Bright, M., Bulgheresi, S., 2010. A complex journey: Transmission of microbial symbionts. Nat Rev Microbiol 8, 218–230. https://doi.org/10.1038/nrmicro2262

Burke, G.R., Normark, B.B., Favret, C., Moran, N.A., 2009. Evolution and diversity of facultative symbionts from the aphid subfamily Lachninae. Appl Environ Microbiol 75, 5328–5335. https://doi.org/10.1128/AEM.00717-09

Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J.A., Holmes, S.P., 2016. DADA2: High-resolution sample inference from Illumina amplicon data. Nat Methods 13, 581–583. https://doi.org/10.1038/nmeth.3869

Cunning, R., Silverstein, R.N., Baker, A.C., 2015. Investigating the causes and consequences of symbiont shuffling in a multi-partner reef coral symbiosis under environmental change. Proceedings of the Royal Society B: Biological Sciences 282. https://doi.org/10.1098/rspb.2014.1725

Darriba, Di., Posada, D., Kozlov, A.M., Stamatakis, A., Morel, B., Flouri, T., 2020. ModelTest-NG: A New and Scalable Tool for the Selection of DNA and Protein Evolutionary Models. Mol Biol Evol 37, 291–294. https://doi.org/10.1093/molbev/msz189

del Campo, J., Kolisko, M., Boscaro, V., Santoferrara, L.F., Nenarokov, S., Massana, R., Guillou, L., Simpson, A., Berney, C., de Vargas, C., Brown, M.W., Keeling, P.J., Wegener Parfrey, L., 2018. EukRef: Phylogenetic curation of ribosomal RNA to enhance understanding of eukaryotic diversity and distribution. PloS Biol 16, e2005849. https://doi.org/10.1371/journal.pbio.2005849

Dettmering, C., Rottger, R., Hohenegger, J., Schmaljohann, R., 1998. The trimorphic life cycle in foraminifera: Observations from cultures allow new evaluation. Eur J Protistol 34, 363–368. https://doi.org/10.1016/S0932-4739(98)80004-7

el Kateb, A., Stalder, C., Stainbank, S., Fentimen, R., Spezzaferri, S., 2018. The genus Amphistegina (Benthic foraminifera): Distribution along the southern Tunisian coast. Bioinvasions Rec 7, 391–398. https://doi.org/10.3391/bir.2018.7.4.06

Esling, P., Lejzerowicz, F., Pawlowski, J., 2015. Accurate multiplexing and filtering for high-throughput amplicon-sequencing. Nucleic Acids Res 43, 2513–2524. https://doi.org/10.1093/nar/gkv107

Flouri, T., Izquierdo-Carrasco, F., Darriba, D., Aberer, A.J., Nguyen, L.T., Minh, B.Q., von Haeseler, A., Stamatakis, A., 2015. The phylogenetic likelihood library. Syst Biol 64, 356–362. https://doi.org/10.1093/sysbio/syu084

Galil, B.S., Goren, M., 2014. Metamorphoses: Bioinvasions in the Mediterranean Sea BT - The Mediterranean Sea: Its history and present challenges, in: Goffredo, S., Dubinsky, Z. (Eds.), . Springer Netherlands, Dordrecht, pp. 463–478. https://doi.org/10.1007/978-94-007-6704-1_27

Gilbert, J.A., Hill, R., Doblin, M.A., Ralph, P.J., 2012. Microbial consortia increase thermal tolerance of corals. Mar Biol 159, 1763–1771. https://doi.org/10.1007/s00227-012-1967-9

Glöckner, F.O., Yilmaz, P., Quast, C., Gerken, J., Beccati, A., Ciuprina, A., Bruns, G., Yarza, P., Peplies, J., Westram, R., Ludwig, W., 2017. 25 years of serving the community with ribosomal RNA gene reference databases and tools. J Biotechnol 261, 169–176. https://doi.org/https://doi.org/10.1016/j.jbiotec.2017.06.1198

González-Pech, R.A., Bhattacharya, D., Ragan, M.A., Chan, C.X., 2019. Genome Evolution of Coral Reef Symbionts as Intracellular Residents. Trends Ecol Evol 34, 799–806. https://doi.org/10.1016/j.tree.2019.04.010

Grégoire, V., Schmacka, F., Coffroth, M.A., Karsten, U., 2017. Photophysiological and thermal tolerance of various genotypes of the coral endosymbiont Symbiodinium sp. (Dinophyceae). J Appl Phycol 29, 1893–1905. https://doi.org/10.1007/s10811-017-1127-1

Guastella, R., Marchini, A., Caruso, A., Cosentino, C., Evans, J., Weinmann, A.E., Langer, M.R., Mancin, N., 2019. "Hidden invaders" conquer the Sicily Channel and knock on the door of the Western Mediterranean sea. Estuar Coast Shelf Sci 225, 106234. https://doi.org/10.1016/j.ecss.2019.05.016

Guillou, L., Bachar, D., Audic, S., Bass, D., Berney, C., Bittner, L., Boutte, C., Burgaud, G., de Vargas, C., Decelle, J., del Campo, J., Dolan, J.R., Dunthorn, M., Edvardsen, B., Holzmann, M., Kooistra, W.H.C.F., Lara, E., le Bescot, N., Logares, R., Mahé, F., Massana, R., Montresor, M., Morard, R., Not, F., Pawlowski, J., Probert, I., Sauvadet, A.-L., Siano, R., Stoeck, T., Vaulot, D., Zimmermann, P., Christen, R., 2013. The Protist Ribosomal Reference database (PR2): a catalog of unicellular eukaryote small sub-unit rRNA sequences with curated taxonomy. Nucleic Acids Res 41, D597-604. https://doi.org/10.1093/nar/gks1160

Hallock, P., Lidz, B.H., Cockey-Burkhard, E.M., Donnelly, K.B., 2003. Foraminifera As Bioindicators In Coral Reef Assessment And Monitoring: The FORAM Index.

Hallock, P., Williams, D.E., Fisher, E.M., Toler, S.K., 2006a. Bleaching in Foraminifera with Algal Symbionts: Implications for Reef Monitoring and Risk Assessment FORAMS 2006. Anuário do Instituto de Geociências -UFRJ 29, 108–128.

Hallock, P., Williams, S.K., Toler, E.M., Fisher, Talge, H.K., 2006b. Bleaching in reef-dwelling foraminifers: implications for reef decline. 10th Int. Coral Reef Symp 737, 729–737.

Harney, J.N., Hallock, P., Talge, H.K., 1998. Observations on a trimorphic life cycle in Amphistegina ilose populations from the Florida keys. J Foraminifer Res 28, 141–147. https://doi.org/10.2113/gsjfr.28.2.141

Herlemann, D.P.R., Labrenz, M., Jürgens, K., Bertilsson, S., Waniek, J.J., Andersson, A.F., 2011. Transitions in bacterial communities along the 2000 km salinity gradient of the Baltic Sea. ISME Journal 5, 1571–1579. https://doi.org/10.1038/ismej.2011.41

Hickling, R., Roy, D.B., Hill, J.K., Fox, R., Thomas, C.D., 2006. The distributions of a wide range of taxonomic groups are expanding polewards. Glob Chang Biol 12, 450–455. https://doi.org/10.1111/j.1365-2486.2006.01116.x

Hottinger, L., Halicz, E., Reiss, Z., 1993. Recent foraminiferida from the Gulf of Aqaba, Red Sea, 33rd ed. Dela SAZU, Ljubljana.

Howells, E.J., Abrego, D., Meyer, E., Kirk, N.L., Burt, J.A., 2016. Host adaptation and unexpected symbiont partners enable reef-building corals to tolerate extreme temperatures. Glob Chang Biol 22, 2702–2714. https://doi.org/https://doi.org/10.1111/gcb.13250

Jones, A., Berkelmans, R., 2010. Potential Costs of Acclimatization to a Warmer Climate: Growth of a Reef Coral with Heat Tolerant vs. Sensitive Symbiont Types. PloS One 5, e10437-.

Joy, J.B., 2013. Symbiosis catalyses niche expansion and diversification. Proceedings of the Royal Society B: Biological Sciences 280, 20122820. https://doi.org/10.1098/rspb.2012.2820

Katoh, K., Standley, D.M., 2013. MAFFT multiple sequence alignment software version 7: Improvements in performance and usability. Mol Biol Evol 30, 772–780. https://doi.org/10.1093/molbev/mst010

Kozlov, A.M., Darriba, D., Flouri, T., Morel, B., Stamatakis, A., 2019. RaxML-NG: A fast, scalable and user-friendly tool for maximum likelihood phylogenetic inference. Bioinformatics 35, 4453–4455. https://doi.org/10.1093/bioinformatics/btz305

Lajoie, G., Parfrey, L.W., 2022. Beyond specialization: re-examining routes of host influence on symbiont evolution. Trends Ecol Evol 37, 590–598. https://doi.org/10.1016/j.tree.2022.03.006

Langer, M.R., 2008. Foraminifera from the Mediterranean and the Red Sea. Aqaba-Eilat, the Improbable GulfEnvironment, Biodiversity and Preservation. The Hebrew University Magnes Press, Jerusalem, pp. 394–415.

Langer, M.R., Hottinger, L., 2000. Biogeography of selected “larger” foraminifera. Micropaleontology 46, 105–126.

Langer, M.R., Mouanga, G.H., 2016. Invasion of amphisteginid foraminifera in the Adriatic Sea. Biol Invasions 18, 1335–1349. https://doi.org/10.1007/s10530-016-1070-0

Lee, J., 2006. Algal symbiosis in larger foraminifera. Symbiosis 42, 63–75.

Lee, J.J., Anderson, O.R., 1991. Symbiosis in Foraminifera, in: Biology of Foraminifera. Academic Press, London, pp. 157–220.

Lee, J.J., Faber, W.W., Nathans, B., Rottger, R., Nishihira, M., Kruger, R., 1992. Endosymbiotic Diatoms from Larger Foraminifera Collected in Pacific Habitats. Symbiosis 14, 265–281.

Leutenegger, S., 1983. Specific Host-Symbiont Relationship in Larger Foraminifera. Source: Micropaleontology 29, 111–125. https://doi.org/https://doi.org/10.2307/1485562

Lynch, M.D.J., Neufeld, J.D., 2015. Ecology and exploration of the rare biosphere. Nat Rev Microbiol 13, 217–229. https://doi.org/10.1038/nrmicro3400

Martin, M., 2011. Cutadapt Removes Adapter Sequences From High-Throughput Sequencing Reads. EMBnet J 17, 10–12. https://doi.org/https://doi.org/10.14806/ej.17.1.200

McMurdie, P.J., Holmes, S., 2013. Phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. PloS One 8, e61217-.

Meriç, E., Avşar, N., Yokeş, M.B., 2008. Some alien foraminifers along the Aegean and southwestern coasts of Turkey. Micropaleontology 54, 307–349.

O'brien, P.A., Webster, N.S., Miller, D.J., Bourne, D.G., 2019. Host-Microbe Coevolution: Applying Evidence from Model Systems to Complex Marine Invertebrate Holobionts. American Society of Microbiology 10, e02241-18. https://doi.org/10.1128/mBio

Oksanen, J., Simpson, G., Blanchet, F., Kindt, R., Legendre, P., Minchin, P., O'Hara, R., Solymos, P., Stevens, M., Szoecs, E., Wagner, H., Barbour, M., Bedward, M., Bolker, B., Borcard, D., Carvalho, G., Chirico, M., de Caceres, M., Durand, S., Evangelista, H., FitzJohn, R., Friendly, M., Furneaux, B., Hannigan, G., Hill, M., Lahti, L., McGlinn, D., Ouellette, M., Ribeiro Cunha, E., Smith, T., Stier, A., ter Braak, C., Weedon, J., 2022. Vegan: Community Ecology Package_. R package version 2.6-2, <https://CRAN.R-project.org/package=vegan>.

Ozer, T., Gertman, I., Kress, N., Silverman, J., Herut, B., 2017. Interannual thermohaline (1979–2014) and nutrient (2002–2014) dynamics in the Levantine surface and intermediate water masses, SE Mediterranean Sea. Glob Planet Change 151, 60–67. https://doi.org/https://doi.org/10.1016/j.gloplacha.2016.04.001

Paradis, E., 2010. Pegas: An R package for population genetics with an integrated-modular approach. Bioinformatics 26, 419–420. https://doi.org/10.1093/bioinformatics/btp696

Peixoto, R.S., Sweet, M., Villela, H.D.M., Cardoso, P., Thomas, T., Voolstra, C.R., Høj, L., Bourne, D.G., 2021. Coral Probiotics: Premise, Promise, Prospects. Annu Rev Anim Biosci 9, 265–288. https://doi.org/10.1146/annurev-animal-090120-115444

Prazeres, M., 2018. Bleaching-associated changes in the microbiome of large benthic foraminifera of the Great Barrier Reef, Australia. Front Microbiol 9, 1–13. https://doi.org/10.3389/fmicb.2018.02404

Prazeres, M., Ainsworth, T., Roberts, T.E., Pandolfi, J.M., Leggat, W., 2017. Symbiosis and microbiome flexibility in calcifying benthic foraminifera of the great Barrier Reef. Microbiome 5, 1–11. https://doi.org/10.1186/S40168-017-0257-7

Prazeres, M., Morard, R., Roberts, T.E., Doo, S.S., Jompa, J., Schmidt, C., Stuhr, M., Renema, W., Kucera, M., 2020. High dispersal capacity and biogeographic breaks shape the genetic diversity of a globally distributed reef-dwelling calcifier. Ecol Evol 10, 5976–5989. https://doi.org/10.1002/ece3.6335

Prazeres, M., Roberts, T.E., Ramadhani, S.F., Doo, S.S., Schmidt, C., Stuhr, M., Renema, W., 2021. Diversity and flexibility of algal symbiont community in globally distributed larger benthic foraminifera of the genus Amphistegina. BMC Microbiol 21, 1–16. https://doi.org/10.1186/s12866-021-02299-8

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., Glöckner, F.O., 2013. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res 41, D590–D596. https://doi.org/10.1093/nar/gks1219

R Core Team, 2022. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

Raposo, D., Zufall, R.A., Caruso, A., Titelboim, D., Abramovich, S., Hassenrück, C., Kucera, M., Morard, R., submitted. Invasion success in a Lessepsian symbiont-bearing foraminifera linked to high dispersal ability, preadaptation and suppression of sexual reproduction. Scientific Reports

Rilov, G., Galil, B., 2009. Marine Bioinvasions in the Mediterranean Sea – History, Distribution and Ecology 549–575. https://doi.org/10.1007/978-3-540-79236-9_31

Rosado, P.M., Leite, D.C.A., Duarte, G.A.S., Chaloub, R.M., Jospin, G., Nunes da Rocha, U., P. Saraiva, J., Dini-Andreote, F., Eisen, J.A., Bourne, D.G., Peixoto, R.S., 2019. Marine probiotics: increasing coral resistance to bleaching through microbiome manipulation. ISME J 13, 921–936. https://doi.org/10.1038/s41396-018-0323-6

Rosenberg, E., Zilber-Rosenberg, I., 2011. Symbiosis and development: The hologenome concept. Birth Defects Res C Embryo Today 93, 56–66. https://doi.org/https://doi.org/10.1002/bdrc.20196

Schmidt, C., Heinz, P., Kucera, M., Uthicke, S., 2011. Temperature-induced stress leads to bleaching in larger benthic foraminifera hosting endosymbiotic diatoms. Limnol Oceanogr 56, 1587–1602. https://doi.org/10.4319/lo.2011.56.5.1587

Schmidt, C., Morard, R., Prazeres, M., Barak, H., Kucera, M., 2016[a]. Retention of high thermal tolerance in the invasive foraminifera Amphistegina lobifera from the Eastern Mediterranean and the Gulf of Aqaba. Mar Biol 163. https://doi.org/10.1007/s00227-016-2998-4

Schmidt, C., Morard, R., Romero, O., Kucera, M., 2018. Diverse internal symbiont community in the endosymbiotic foraminifera Pararotalia calcariformata: Implications for symbiont shuffling under thermal stress. Front Microbiol 9, 1–11. https://doi.org/10.3389/fmicb.2018.02018

Schmidt, C., Titelboim, D., Brandt, J., Herut, B., Abramovich, S., Almogi-Labin, A., Kucera, M., 2016b. Extremely heat tolerant photo-symbiosis in a shallow marine benthic foraminifera. Sci Rep 6, 1–9. https://doi.org/10.1038/srep30930

Sorgente, R., Drago, A.F., Ribotti, A., 2003. Seasonal variability in the Central Mediterranean Sea circulation.

Stoeck, T., Bass, D., Nebel, M., Christen, R., Jones, M.D.M., Breiner, H.W., Richards, T.A., 2010. Multiple marker parallel tag environmental DNA sequencing reveals a highly complex eukaryotic community in marine anoxic water. Mol Ecol 19, 21–31. https://doi.org/10.1111/j.1365-294X.2009.04480.x

Stuhr, M., Meyer, A., Reymond, C.E., Narayan, G.R., Rieder, V., Rahnenführer, J., Kucera, M., Westphal, H., Muhando, C.A., Hallock, P., 2018. Variable thermal stress tolerance of the reef-associated symbiont-bearing foraminifera Amphistegina linked to differences in symbiont type. Coral Reefs 37, 811–824. https://doi.org/10.1007/s00338-018-1707-9

ter Kuile, B., Erez, J., 1988. The size and function of the internal inorganic carbon pool of the foraminifer Amphistegina lobifera. Mar Biol 99, 481–487. https://doi.org/10.1007/BF00392555

Titelboim, D., Almogi-Labin, A., Herut, B., Kucera, M., Asckenazi-Polivoda, S., Abramovich, S., 2019. Thermal tolerance and range expansion of invasive foraminifera under climate changes. Sci Rep 9, 1–5. https://doi.org/10.1038/s41598-019-40944-5

Triantaphyllou, M. v., Dimiza, M.D., Koukousioura, O., Hallock, P., 2012. Observations on the life cycle of the symbiont-bearing foraminifer Amphistegina lobifera Larsen, an invasive species in

coastal ecosystems of the ilose sea (Greece, E. Mediterranean). J Foraminifer Res 42, 143–150. https://doi.org/10.2113/gsjfr.42.2.143

Vinje, H., Liland, K.H., Almøy, T., Snipen, L., 2015. Comparing K-mer based methods for improved classification of 16S sequences. BMC Bioinformatics 16. https://doi.org/10.1186/s12859-015-0647-4

Walther, G.-R., Post2, E., Convey3, P., Menzel, A., Parmesank, C., Beebee, T.J.C., Fromentin#, J.-M., Hoegh-Guldberg, O., Bairlein, F., 2002. Ecological responses to recent climate change.

Webster, N.S., Reusch, T.B.H., 2017. Microbial contributions to the persistence of coral reefs. ISME J 11, 2167–2174. https://doi.org/10.1038/ismej.2017.66

Weiner, A.K.M., Morard, R., Weinkauf, M.F.G., Darling, K.F., André, A., Quillévéré, F., Ujiie, Y., Douady, C.J., de Vargas, C., Kucera, M., 2016. Methodology for single-cell genetic analysis of planktonic foraminifera for studies of protist diversity and evolution. Front Mar Sci 3, 1–15. https://doi.org/10.3389/fmars.2016.00255

Yilmaz, P., Parfrey, L.W., Yarza, P., Gerken, J., Pruesse, E., Quast, C., Schweer, T., Peplies, J., Ludwig, W., Glöckner, F.O., 2014. The SILVA and "All-species Living Tree Project (LTP)" taxonomic frameworks. Nucleic Acids Res 42, D643–D648. https://doi.org/10.1093/nar/gkt1209

Yokes, M.B., Meric, E., Avsar, N., 2007. On the Presence of alien foraminifera Amphistegina lobifera Larsen on the coasts of the Maltese Islands. Aquat Invasions 2, 439–441. https://doi.org/10.3391/ai.2007.2.4.15

Zhang, Ying, Yang, Q., Ling, J., Long, L., Huang, H., Yin, J., Wu, M., Tang, X., Lin, X., Zhang, Yanying, Dong, J., 2021. Shifting the microbiome of a coral holobiont and improving host physiology by inoculation with a potentially beneficial bacterial consortium. BMC Microbiol 21, 130. https://doi.org/10.1186/s12866-021-02167-5

Ziegler, M., Seneca, F.O., Yum, L.K., Palumbi, S.R., Voolstra, C.R., 2017. Bacterial community dynamics are linked to patterns of coral heat tolerance. Nat Commun 8, 14213. https://doi.org/10.1038/ncomms14213

Chapter 3

Physiological adaptation of invasive tropical symbiont-bearing foraminifera to low-temperature (winter conditions) in the Mediterranean Sea

This work is in preparation for submission.

Débora S. Raposo*[1], Christiane Schmidt[2], Christiane Hassenrück[1,3], Danna Titelboim[4], Sigal Abramovich[5], Raphaël Morard[1], Michal Kucera[1]

[1] MARUM, Center for Marine Environmental Sciences, Universität Bremen, Bremen, Germany

[2] Leibniz Centre for Tropical Marine Research (ZMT), Bremen, Germany

[3] Leibniz Institute for Baltic Sea Research Warnemünde (IOW), Rostock-Warnemünde, Germany

[4] Department of Earth Sciences, University of Oxford, Oxford, United Kingdom

[5] Department of Earth and Environmental Sciences, Ben Gurion University of the Negev, Beer Sheva, Israel

Data availability: All data generated in this study will be available on the public repository PANGAEA Data Publisher once the paper is published.

ABSTRACT

The invasive diatom-bearing foraminifera *Amphistegina lobifera* has become abundant in its newly conquered Mediterranean habitats. There, it is exposed to colder winters (~13°C) and warmer summers (31°C) that exceed the range of its native habitat in the Red Sea (22 °C in winter to 28 °C in summer) and potentially its Indo-Pacific origin. To understand how the species spread its range despite the higher seasonality in the invaded habitat, it is of critical importance to understand the mechanisms that facilitated its invasion success. To this end, we examined the physiological response of *A. lobifera* to cold temperatures for the source population (Red Sea), early invader population (Eastern Mediterranean), and invasion front population (Sicily) to a range of low temperatures (10, 13, 16, 19°C) to assess whether the population has higher adaptive range than already displayed, compared towards the control treatment of 25°C. We conducted a four-week experiment in which we monitored the physiological response of the host (growth, motility) and the photosynthetic activity of the symbionts by Pulse Amplitude Modulation (PAM) fluorometry. All foraminifera hosts showed a reduced growth rate < 19°C and no motility (pseudopodia movement) below 13°C compared to the control. The photo-physiological response of the symbionts was different. The Red Sea and Eastern Mediterranean populations showed reduced Fv:Fm at colder temperatures, whereas the symbionts of the Sicily population maintained a high photosynthetic activity even in the coldest treatments. Since the host response did not differ across the tested range of temperatures, but the photo-physiology of the symbionts in Sicily was significantly different from the other populations we infer the following. The adaptive success of the invasive population could be related to a different set of symbionts rather than an adaptation of the host. This suggests that cold-tolerant photosymbionts or the flexibility to form symbioses with differently adapted algae is a key to the success of past and future migrations.

Keywords: Larger benthic foraminifera, photosymbiosis, physiological response, Lessepsian invasion

1. INTRODUCTION

Biotic invasions are one of the most severe threats to biodiversity conservation (Bellard et al., 2016; Simberloff et al., 2013) and one of the major human-driven global changes (Mack et al., 2000). When colonizing new habitats, invasive species are faced with climatic (seasonality),

physical (light), chemical (salinity), and biotic (microbiome and interactome) parameters that exceed the range they experience within their native habitat. This results in either retention, expansion, or reduction of their ecological niche (i.e., the set of all biotic and abiotic conditions in which a species is observed in nature) (Guisan and Thuiller, 2005; Pearman et al., 2008; Wiens et al., 2009). That is because species ranges are limited by their physiological tolerances (which define the fundamental niche), as well as the biotic interactions and dispersal barriers (which constrain the realized niche, i.e., the actual space that an organism inhabits and the resources it can access) (Tingley et al., 2014). To accurately predict biological invasions it is, therefore, crucial to understand if species conserve their native niche (niche conservatism; Liu et al., 2020; Wiens and Graham, 2005) or if fundamental and realized niche shifts occur. Niche shifts could be caused by either the result of evolved environmental tolerance or the development of relevant attributes that can aid the adaptation of a species to the foreign conditions (fundamental niche shifts) or the presence of novel biotic and abiotic conditions in the invaded range (realized niche shifts) (Pearman et al., 2008; Tingley et al., 2014).

A particular invasive phenomenon, known as the Lessepsian Invasion, in which the invaded species are exposed to a wide range of different environmental conditions has taken place in the last c.a. 150 years. Over 600 tropical Indo-Pacific species from the Red Sea have made their way into the Mediterranean Sea since the Suez Canal was opened and connected the two systems (Rilov and Galil, 2009; Zenetos et al., 2012). There, the invaders must contend with both warmer summer (31°C) and lower winter temperatures (13 °C) (Sorgente et al., 2003) that are outside the range experienced in their source location in the Red Sea where the temperature is buffered throughout the year (22 °C in winter to 28 °C in summer, Schmidt et al. 2016). Among the most successful Lessepsian invaders is the large benthic foraminifera (LBF) *Amphistegina lobifera*, a diatom-bearing species (Alve and Goldstein, 2003). After a few decades of gradual north and westward migration (Caruso and Cosentino, 2014; el Kateb et al., 2018; Langer et al., 2012; Langer and Mouanga, 2016; Meriç et al., 2008; Schmidt et al., 2016; Triantaphyllou et al., 2012; Yokes et al., 2007), this prolific calcifier has recently reached its invasion front in Sicily (Guastella et al., 2019; Raposo et al., submitted). Intriguingly, even though *A. lobifera* is regarded to prefer warmer tropical conditions (Hyams et al., 2002), the invasive population became dominant in Sicily, sometimes exceeding 50% of the assemblages after only three years since its first appearance (Raposo et al., submitted).

The thermal tolerance of LBFs is closely connected to their relationship with their algae symbionts (Hallock et al., 2006b), and the foraminifera host relies on them to enhance growth

and calcification (Lee and Anderson, 1991). Such novel conditions experienced in the invaded ecosystem have the potential to disrupt the community interactions that contribute to the fitness of the holobiont (i.e., host and associated microbiome) (Greenspan et al., 2019). Thus, to assess the holobiont fitness during invasion the microbiome structure and its physiological response to the environmental changes needs to be taken into account. Previous investigations have found that the invasive *A. lobifera* populations carried a distinct and more diverse diatom community than the source population (Prazeres et al., 2021; Raposo et al., in prep.). However, whether these different symbionts are responsible for their adaptation to the new environment, is not yet fully understood. The recent findings of Titelboim et al. (2021) advanced our knowledge in this sense, by revealing that under cold stress (15°C), cellular resources of invasive *A. lobifera* are conveyed to the maintenance of photosynthesis with the cost of decelerating the growth of the foraminifera host. This study thus suggests that the symbionts play a significant role in the physiological adaptation and raises the question of whether the higher performance of the symbionts is inherited from the source population or if it is a result of an adaptation gained post-invasion.

On the warmer end (30-32°C), the tolerance of the *A. lobifera* host and its symbionts is more documented, and it is believed to be a conservative trait retained from the source population (Schmidt et al., 2016; Titelboim et al., 2019). Thus, the invasive populations are favored by a pre-adaptation to high temperatures even though it is not "needed" in their native setting (Schmidt et al., 2016). Such an example of niche conservatism is the fundament of niche modeling and niche-model-based projections of range expansions under future change scenarios (Liu et al., 2020; Wiens et al., 2009). However, whether the success of the invasion of *A. lobifera* in the colder temperatures at the invasion front is also related to pre-adaptation (as observed at the warm end) or to their different symbiotic associations is not yet resolved. For this, here we test the niche stability hypothesis by analyzing the thermal tolerance of invasive and source populations of the foraminifera species *Amphistegina lobifera* and their associated diatoms. These foraminifera are a suitable model for this test because of their well-documented history of invasion into the Mediterranean.

Our aim was to determine (a) if the sustained exposure to winters ~9°C colder than in the Red Sea has induced adaptations in the foraminiferal holobiont, which could explain its successful and rapid west-ward invasion in the Mediterranean Sea; or (b) if the foraminiferal capacity to withstand cold winters is a matter of pre-adaptation, being inherited from the source population. For this, we conducted a physiological experiment in which we analyzed the thermal tolerance

of a well-established invasive population in Haifa, Israel (Eastern Mediterranean) and a population at the invasion front (Sicily), and compared those with the source population (Red Sea). We analyzed the physiological responses of the host (growth, motility) and their diatoms (photosynthetic activity, Chlorophyll *a*).

2. MATERIAL AND METHODS

2.1 Collection and maintenance of living *A. lobifera*

Three populations of *A. lobifera* were sampled to recover different stages of the Lessepian invasion (Figure 1). Living specimens were collected in Eilat (Red Sea) which represents the source population, in Haifa (Eastern Mediterranean) sea which represents the pioneer invading population, and in Sicily (Western Mediterranean) which represents the front of the invasion. The three locations were sampled between September and October 2019 when the water temperature was ~26°C at the three locations (Table 1).

Table 1 Location of sampling stations of living *Amphistegina lobifera* specimens

Station	Date	Location	Lat	Long	Depth	Seawater Temperature	Salinity	Remarks
Sicily	21.09.2019	Capo Passero	36.6865	15.1381	2.0 m	26.0 °C	38.0 ‰	Front of invasion
East Med	23.10.2019	Tel Shikmona, Haifa	32.8258	34.9548	1.5 m	25.6 °C	39.7 ‰	Pioneer invaders
Red Sea	27.10.2019	Gulf of Aqaba	29.502	34.9179	5.0 m	25.7 °C	40.4 ‰	Source population

The sampling and processing at the lab station were conducted according to the protocol proposed by (Hallock et al., 2006a) and previous studies by (Schmidt et al., 2016; Stuhr et al., 2018). Macroalgae and pebbles where the foraminifera live were collected by snorkeling, carried to the shore and brushed in a bucket filled with ambient seawater. The obtained sediment was sieved (150 – 2000 µm), transferred into several 0.5 L plastic jars filled with seawater, and transported to the field laboratory in insulated bags. Under a stereomicroscope, c.a. 1500 living specimens of *A. lobifera* per location were picked from the sieved sediment to ensure that we would have enough individuals to conduct the experiment, but the sieved sediment was kept for further use as stock culture. The picked specimens were put in 100 mL screw-capped plastic jars previously filled with filtered (0.20µm) seawater with a maximum of 50 specimens per jar to avoid a severe decrease in dissolved oxygen and mitigate the risk of

container failure during transport. The jars with the picked specimens (~30 jars per location) as well as the jars with the stock culture (1 jar per location) were shipped to Germany (shipment duration < 72 h) in insulated boxes. For each shipment, the temperature was recorded in a jar containing only seawater and a HOBO logger (Onset, USA) (Table S1 for temperature records during shipment). All jars were half-filled with new filtered seawater before shipment.

After the reception of the samples at MARUM (Bremen, Germany), the specimens were transferred together with the stock culture to ensure their survival for longer periods by keeping their natural microbiota. They were kept in culture tanks (2 L) filled with artificial seawater at 38.5‰ salinity (Tropic Marine® Sea Salt Classic) and placed in incubators at 25°C +- 0.5°C with 12h/12h diurnal cycle (<40 µmol photons $m^{-2}s^{-2}$), with air pumps switching on twice a day for 30 min for oxygenation. To avoid evaporation and an increase of salinity, all tanks were sealed with Parafilm®. Temperature and salinity measurements were controlled weekly, followed by ½ water exchange. The cultures were fed monthly with autoclaved algae *Nannochloropsis* food mixture (Schmidt et al., 2016). The cultures were kept in these conditions for a few weeks to acclimatize and recover from handling and shipment before the beginning of the experiment. The Sicilian culture was kept four weeks longer in these conditions until the end of the acclimation period of the specimens from Israel, which were collected at a later date.

2.2 Experiment setup

The experiment was designed to observe the fitness of each population at the minimal temperature faced by the invasive population. We measured the physiological responses of the host (growth rate, motility) and their symbionts (photosynthetic activity, Chl α).

The *Amphistegina lobifera* specimens for the experiment were picked from the stock cultures after the end of their recovery phase of at least four weeks. We selected specimens (300 per population) with regular brown color and showing pseudopodia activity (Prazeres et al., 2016) above 0.5 µm to make comparisons between identical life stages and to avoid selecting by error specimens of the sister species *A. lessonii* that are not distinguishable from smaller *A. lobifera* and occured in the Red Sea samples.

Throughout four weeks (20 Nov-19 Dec 2019), we exposed each population to four treatments (10, 13, 16, and 19 °C) and a control temperature (25°C). For each treatment, two replicate tanks (1L) were set-up per population, holding six small housing filled with five foraminifera specimens each (sub-replicates) (Figure 1). The housings were closed by a 100 µm mesh to

avoid specimens escaping while allowing water exchange. All measurements were based on the average of the specimens per housing since it is not possible to track the individual specimen's responses.

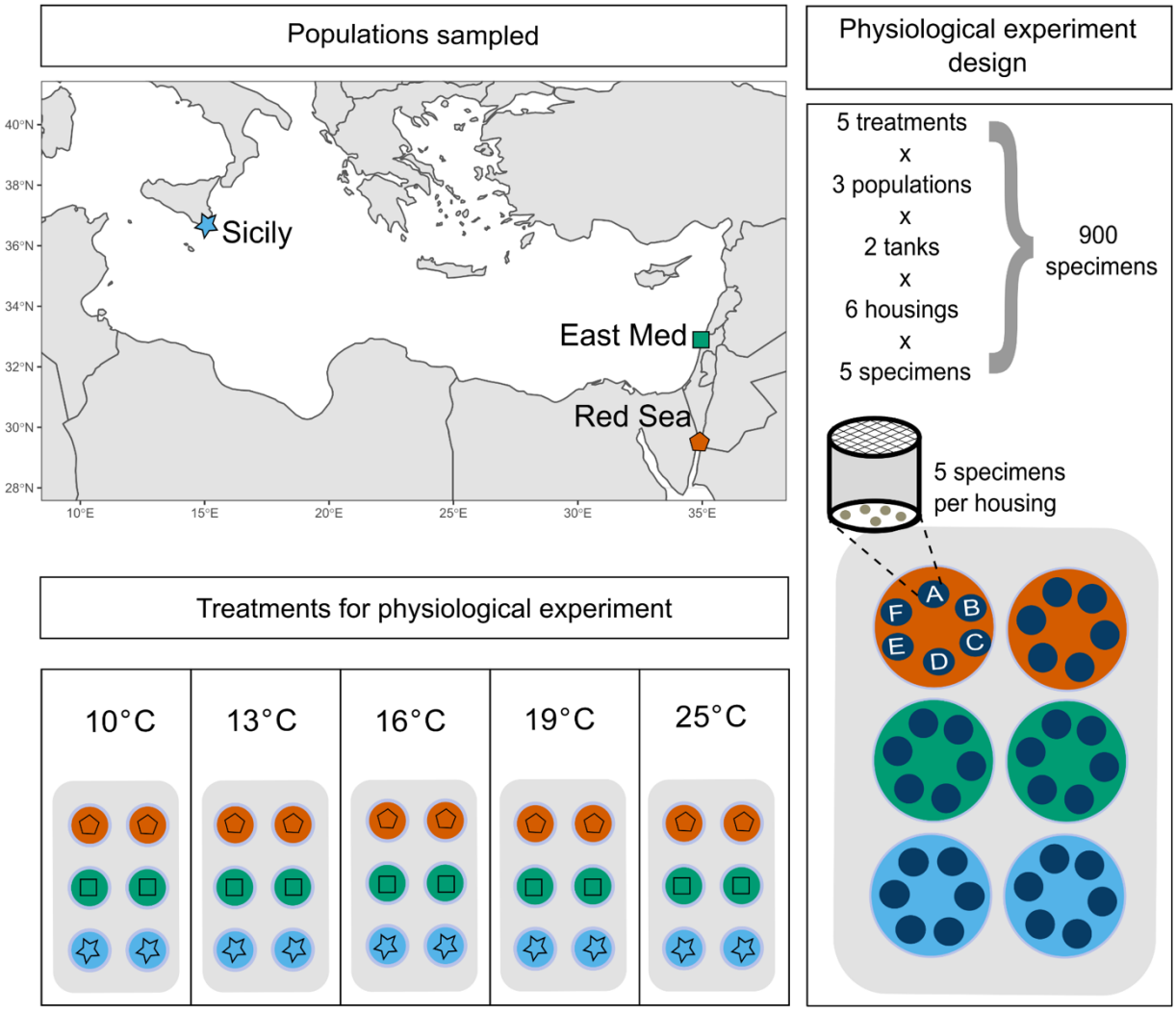


Figure 1. *Populations of Amphistegina lobifera sampled across the Lessepsian invasion range (Gulf of Eilat – source population from the Red Sea (red); Tel Shikmona – pioneer invaders in the Eastern Mediterranean (green); and Sicily – invasion front (blue) and exposed to a cold stress physiological experiment (10, 13, 16 and 19°C) and a control treatment (25°C).*

Lighting was provided by lamps inside the incubators (Philips TL Mini 8W/840) and photosynthetic active radiation (PAR) measurements were taken with a quantum meter (Apogee MQ-200, USA). The tanks placed in the middle were receiving ca. 15 µmol photons $m^{-2}s^{-2}$ while the ones in the edges were receiving ca. 35 µmol photons $m^{-2}s^{-2}$. To make sure all populations received the same light throughout the entire experiment, the positions of the tanks were changed every three days. Oxygenation was provided by pumps programmed to switch

on twice a day for 30 min. With a hand-held probe (WTW, Germany), salinity and temperature were measured daily (Figure S1) and, with HOBO temperature loggers (Onset, USA) installed per incubator, temperature was measured hourly (Table S2). Every week half of the tank volume was exchanged by new artificial seawater at the same temperature of the treatment and each housing received 30 µL of the autoclaved *Nannochloropsis* food mixture.

2.3 Holobiont physiological response: Growth and motility measurements

To monitor the fitness of the host during the experiment, we measured the motility and growth of the foraminifera. Motility is an index of foraminifera pseudopodia activity (Schmidt et al., 2011) as they actively move by means of their pseudopodial network which allows the specimens to climb and attach to the walls of the glass housings. In this study, the motility was measured by counting the number of specimens (from 0 to 5) that were motile (climbing the walls). The specimens were placed back in the bottom once per week and the motility was checked once to twice weekly (Figure 2).

Growth measurements were based on pictures registered at the beginning of the experiment (before the temperature drop) and at the end of the experiment (stereomicroscope V8 Zeiss with Canon EOS 600D, magnification 2.0). The images were processed in the free software ImageJ bundled with 64-bit Java 1.8.0_112 (Schneider et al., 2012) to obtain the surface areas (mm^2) of the specimens, then the average per housing (mean of the five specimens) was obtained. Finally, for each housing we calculated the increase in the surface area in comparison with the initial size and divided by the number of days between the two measurements to estimate the growth rate (% surface area x day $^{-1}$) (Figure 2).

2.4 Physiological response of the symbionts: PAM fluorometry

To assess the performance of the diatom symbionts we investigated the photochemical efficiency of the photosystem II (PSII) by pulse amplitude modulated (PAM) fluorometry. Measurements were carried out once a week, using an IMAGING-PAM Fluorometer (M-Series, WALZ GmbH, Germany), with the "MAXI-Head,1/2" CCD camera equipped with LED lights and a zoom objective (F1.0/f = 8–48 mm). For every housing, the five specimens were taken together with the treatment seawater and placed in small petri dishes, in which the measurements were conducted. After 15-30 min of dark adaptation, the maximum quantum yield (F_v:F_m) was measured, calculated as the ratio between the maximum fluorescence (Fm) and the variable fluorescence (Fv). Next, we measured the light-adapted response (effective quantum yield, Y(II)) which is obtained after the exposition to a light beam at 20 µmol photons

$m^{-2}s^{-1}$ (as the experimental light conditions) for 30 seconds duration (Schmidt et al., 2016) (Figure 3). The detailed weekly measurements of all parameters are reported in Tables S5-S8 in the Supplementary material.

2.5 Chlorophyll α measurements

We used the Chl *a* as a proxy for symbiont biomass (Schmidt et al., 2011) to evaluate how much the diatom density varied across the different temperatures. For this measurement, all five specimens per housing were placed inside 1.5 mL Eppendorf, air dried in the dark, and frozen at -80 °C. For each treatment and population, two housings were analyzed from each tank. Additionally, 2x 5 specimens of each populations from the stock cultures at the beginning of the experiment were measured for initial Chl *a* levels. The Chl *a* was extracted following the method described by Schmidt et al., (2011). In summary, the specimens were crushed in absolute ethanol, incubated at 80°C for 5 min, and left at 4°C for 24h to complete extraction. Chl *a* was quantified in a microplate spectrophotometer (Epoch, BioTek, USA) (absorbances at 665 and 750 nm) and standardized by the size (average surface area) of the specimens.

2.6 Statistical analyses

For the statistical analyses, we separated the observed variables into two categories: binary motility, because the result is either yes-motile or no-nonmotile, and continuous (growth rate, Chl *a*, F_v:F_m, Y(II)). To test if the motility of foraminifera was significantly different for the different foraminifera populations (Population), temperature treatments (Temperature), and sampling time points (Week), we ran a logistic regression with a generalized linear mixed model fit by maximum likelihood (Laplace approximation). We defined tank as the random effect in the model as it was the blocking unit of the experiment. This analysis was performed in R (R Core Team, 2022) with the package “lme4” (Bates et al., 2015). Because of technical issues, we don’t have the data of motility at 19°C of week 4, therefore two models were run (one model with all four weeks – without the 19°C treatment, and the other model without the Week 4 – with all treatments). Significant differences among the factors Population, Temperature, and Week were summarized in Table 2. To test for significance within factor levels, post-hoc contrast analyses were performed (Table S3).

For the remaining variables with continuous data we conducted general linear mixed-effects models to test if the physiological responses were significantly different among foraminifera populations, temperature treatments, and sampling time points (weeks) using the same model formula as before. P-values were adjusted for multiple testing by the false discovery rate "FDR"

method (summarized in Table 3 and shown as relevant letters in Figure 3, Figure 5, and Figure S2). We transformed the data to follow the normal distribution assumptions when necessary and we conducted permuted (1000 times) post-hoc tests, which reduces the effects of slightly not normal data. In case of loss of a specimen or negative growth (e.g. breaking of the shell during manipulation), the whole housing was disregarded in the statistics, to avoid false results caused by erroneous inferences. For the photochemistry, F_v:F_m and Y(II) $\leq$ 0.1 represents severe damage in the photosystem (Schmidt et al., 2016) and therefore were removed from the dataset before these analyses. The analyses were performed in R (R Core Team, 2022) with the packages "nlme" (Pinheiro et al., 2022; Pinheiro and Bates, 2000) and "predictmeans" (Luo et al., 2021). The datasets are available in the Supplementary material.

3. RESULTS

We successfully observed 900 *A. lobifera* specimens every week between 20.11 and 19.12.2019 to evaluate the foraminifera host's response (motility) and the photosynthetic activity of the symbionts (F_v:F_m, Y(II)) to cold stress. After the experiment was terminated, the growth rate of the host and symbiont biomass (Chl *a*) were effectively obtained.

3.1 Holobiont physiological response

The host motility (pseudopodial activity) reduced expressively in all populations with temperature decrease, being clear in the treatments below 13°C by Week 2, when it dropped to ~zero remaining at this level over the course of the experiment (Figure 2). The motility at the higher temperatures did not show a visible trend as they kept oscillating. The different populations showed similar motility responses as further confirmed by the analysis of the two models tested (without 19°C treatment and without 4th week). In both models, the effects of Time and Temperature factors were stronger than the Population factor (Table 2).

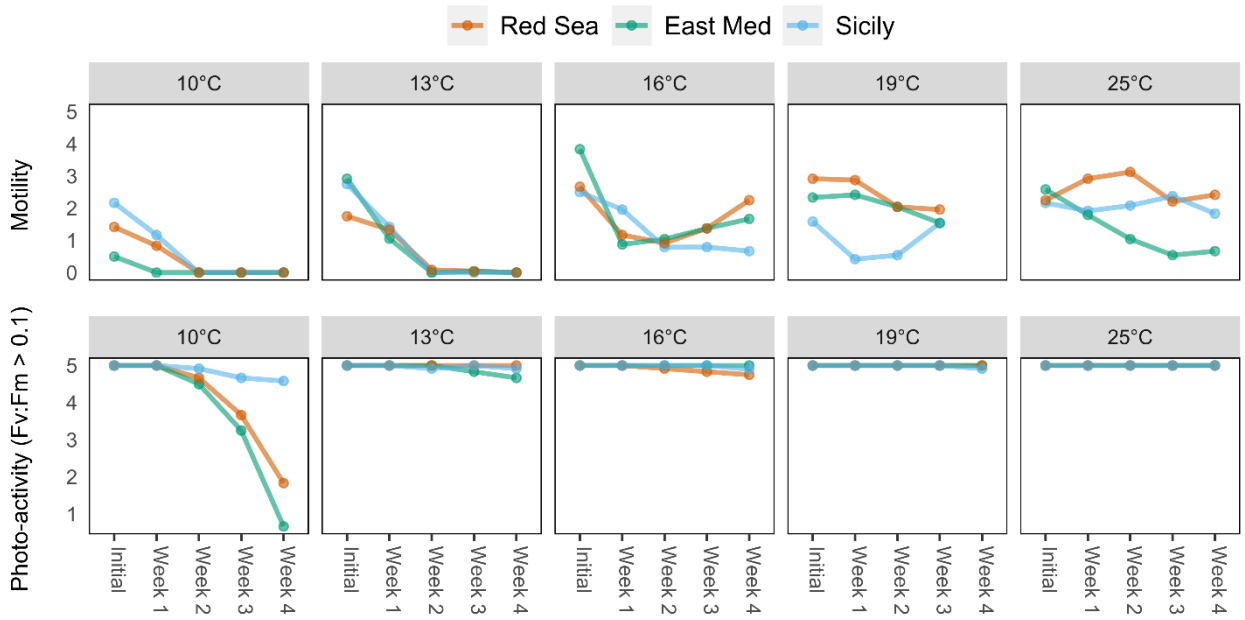


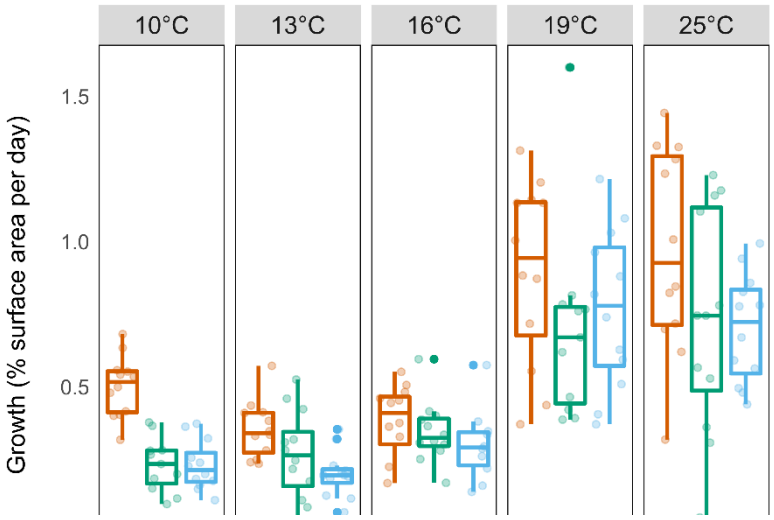
Figure 2. *Motility (index of pseudopodia activity specimens) and Photo-activity (F_v:F_m > 0.1, index of photosystem health) response to thermal stress in the four weeks experiment with Amphistegina lobifera from two invasive populations (Sicily, East Med) and the source population (Red Sea). The y-axes range from 0 (no specimen in the housing was motile / showed photo-activity) to 5 (all specimens were motile / showed photo-activity). Each point in the line plots represents the average of 12 housings. "Initial" refers to the measurement before the experiment was started.*

Table 2 Analysis of variance of the binary variable (motility) to test for significance of each factor: temperature (Temp), population (Pop) and time (Week). Generalized mixed models (1) and (2) fit by maximum likelihood (Laplace approximation), family binomial (logistic regression).

Motility	*(1) All temperatures*			*(2) All weeks*		
	Df	F value		Df	F value	
Time (Week)	3	25.24	*	4	16.35	*
Population (Pop)	2	3.15		2	7.41	
Temperature (Temp)	4	19.16		3	30.44	
Week:Pop	6	3.23	*	8	4.43	*
Week:Temp	12	8.02	*	12	7.13	*
Pop:Temp	8	8.28	*	6	9.27	*
Week:Pop:Temp	24	3.22	*	24	2.23	*

The colder temperatures also affected the growth rates. All three populations showed lower growth below 16°C (Figure 3). Both Temperature and Population factors were significant, and

Temperature had a stronger effect than Population in the growth response (Table 3). We observed a higher growth of the Red Sea population, yet these differences were not statistically significant within each treatment level. The post-hoc test reveals statistically significant differences between the colder (10, 13 and 16°C) and the warmer temperatures (Table 3).



***re 3.** Growth rate in onse to temperature stress i our weeks experiment wit histegina lobifera from tw sive populations (Sicil n in Blue, East Med show Green) and the sourc lation (Red Sea shown i . Growth rate (% surfac increase per day esented as boxplots (n = 1 points, each data poir esents the average of the fiv imens in their housing)*

Table 3 Analysis of variance of the continuous variables (growth rate square root, Chl *a*, F_v:F_m and Y(II)) generalized mixed effects models to test for interactions between the fixed effects: temperature (Temp), population (Pop) and time (Week). Different letters indicate significant differences based in $p < 0.05$ (p-value adjusted for multiple comparisons by the false discovery rate "FDR" method

hl a	Temperature (Te	5	18	0.001	10-A 13-B 16-B 19-C 25-A initial-A
	Population (Pop	2	15	0.001	Red Sea-A East Med-B Sicily-C
					See Figure S2 for stats per week
	Temp x Pop x W	32	787	0.001	

Y(II)	Temperature (Temp)	4	15	26.61	0.001	10-A 13-B 16-B 19-C 25-C
	Population (Pop)	2	15	69.93	0.001	Red Sea-A East Med-B Sicily-C
	Time (Week W)	4	787	236.80	0.001	Initial-A W1-B W2-C W3-D W4-E
	Temp x Pop	8	15	4.22	0.001	
	Temp x Week	16	787	31.03	0.001	See Figure S2 for stats per week
	Pop x Week	8	787	52.51	0.001	
	Temp x Pop x Week	32	787	5.44	0.001	

3.2 Symbionts physiological response

The symbionts photochemical performance to the 4-week experiment diverged according to time, population, and temperature. Before the temperature drop, the maximum quantum yield (F_v:F_m) was similar for all populations (> 0.6). After 4-weeks exposure, the Sicily symbionts stood out with the highest F_v:F_m at the 10 and 13 °C treatments and very little variation across time for all treatments (Figure 4, see Figure S2 for results per week). Meanwhile, the East Med and Red Sea populations clearly showed a reduction in the F_v:F_m at colder temperatures. Significative differences in the populations' performances were observed at 10°C and 13°C, while in the other treatments the three populations remain with similar levels (significance demonstrated by the different letters in Figure 4 after running the post hoc test for the interaction between the factors Temperature, Population and Time). The analysis of the mixed effects model suggests that Time and Temperature are the stronger factors in the F_v:F_m response (Table 3). The average of specimens per housing showing photosynthetic activity (F_v:F_m > 0.1, an index of photosystem health) was strongly reduced at 10°C for the Red Sea and East Med populations, where values of F_v:F_m were often below 0.1 or "not detected" (Figure 2).

The effective quantum yield (Y(II)) was significantly different between the populations in the beginning of the experiment (Figure 4) and after the 4 weeks of exposure, the Sicily symbionts were prominent with the highest Y(II) in all treatments. The symbionts from the East Med and Red Sea populations however showed significant reduction in Y(II) at 13 and 16°C and even lower at 10 °C when compared to the performance of the symbionts from the invasion front. The analysis of the model suggests that the effect of the factors Time and Population are stronger than Temperature in the Y(II) response. For both F_v:F_m and Y(II) models, Time is the factor with the strongest effect (Table 3).

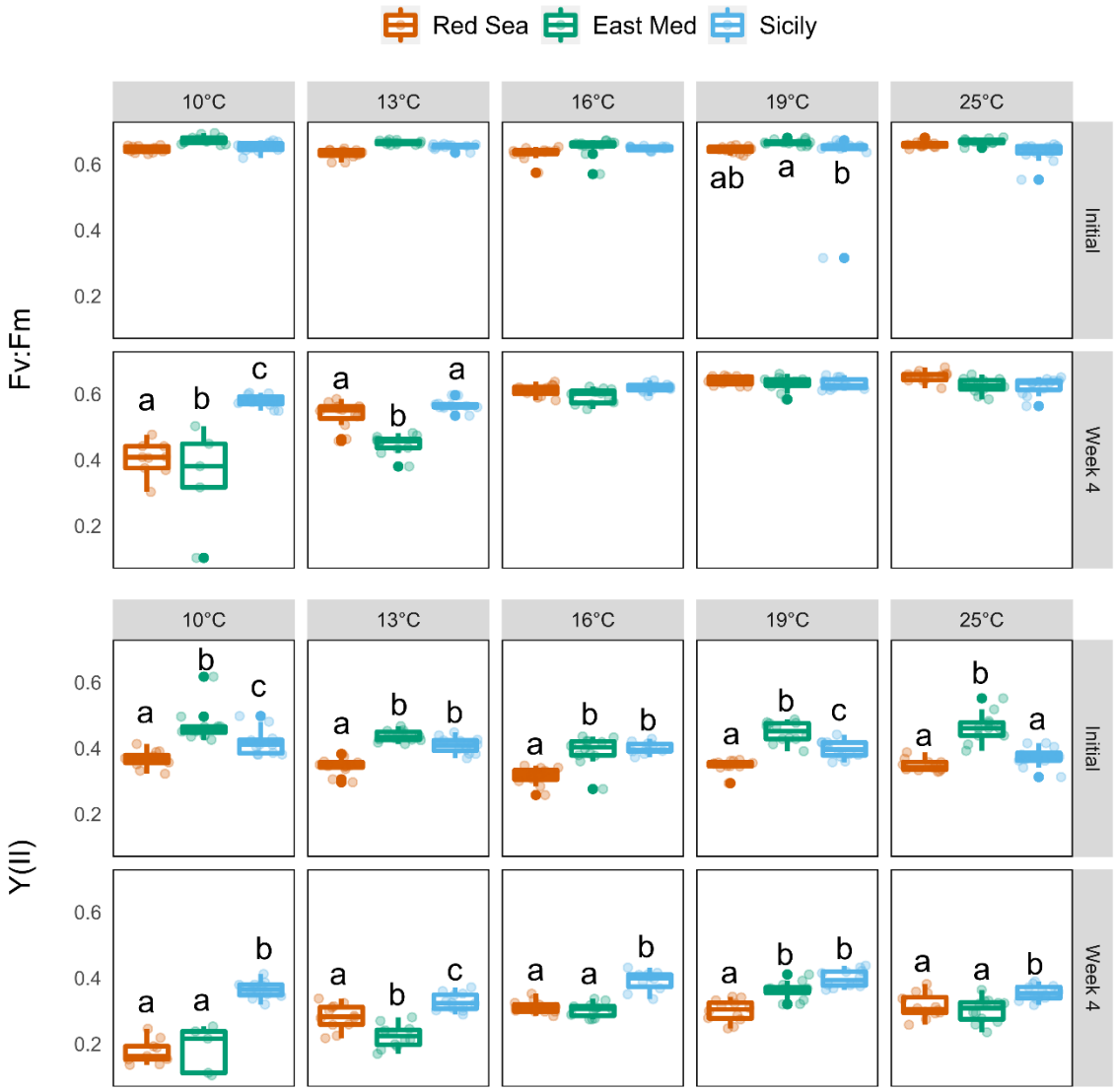
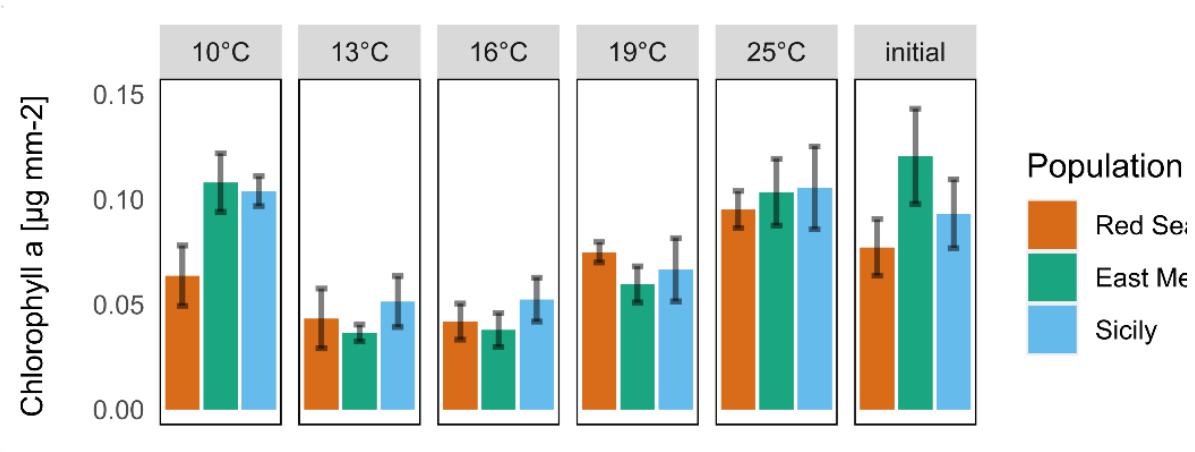


Figure 4. *Photochemistry responses (dark adapted F_v:F_m and light adapted Y(II)) of Amphistegina lobifera symbionts from two invasive populations (Sicily, East Med) and the source population (Red Sea) exposed to temperature stress. "Initial" refers to the measurement before the experiment was started. Different letters indicate significant differences based in $p < 0.05$ (p-value adjusted for multiple comparisons by the false discovery rate "FDR" method). See Figure S2 for detailed photochemistry analysis for each week.*

The Chl *a* (proxy for symbionts biomass) continuously reduced with a decrease in temperature, with exception of the 10°C treatment where the invasive populations showed comparable symbiont biomass to the ones observed in the initial levels and in the control treatment (Figure 5, see Table 3 for detailed statistical significance). In all treatments, Red Sea showed lower values of Chl *a* when compared to the invasive populations.



experiment with Amphistegina lobifera from two invasive populations (Sicily, East Med) and source population (Red Sea). Results showed as bar plots (each bar represents the average housings +/- the SE). "Initial" refers to the measurement before the experiment was started.

4. DISCUSSION

Our physiological experiment allowed us to investigate whether the *Amphistegina lobifera* populations and their diatom symbionts carry similar adaptations to cope with extreme winter temperatures as compared to the ones experienced in their native location or if the invasive populations possess specific adaptations allowing them to succeed in the new condition.

All populations started the experiment with a healthy photosystem (F_v:F_m > 0.6) and their efficiency decreased only in the lower temperatures (10 and 13°C). Even though Temperature and Time accounted for the strongest effects in the statistical analysis, the Population factor still played a role in the models, mainly when we observe the results of the effective quantum yield, Y(II). The initial values of Y(II) were markedly different among the populations, despite all of them having started with a similar maximum quantum yield, F_v:F_m (Figure 4). This variable performance on the symbionts' photo efficiency even in non-stressful conditions is consistent with previous physiological studies on this species (Schmidt et al., 2016), and could be explained by their flexible diatoms associations across the invasive range (Raposo et al., in prep.). Therefore, their different symbiosis associations lead to different levels of photosynthetic performances of the holobiont that is perceptible already in control/initial conditions and even more pronounced under cold stress.

Our physiological experiment's foremost finding is that the *A. lobifera* population in the invasion front (Sicily) harbors better cold-adapted diatoms. Their diatoms showed the highest

values of both F_v:F_m and Y(II) under cold stress (≤ 13°C) since Week 1 (Figure S2) and they sustained a healthy performance until the end of the experiment while the performances of the other populations drastically dropped (Figures 2, 4). Interestingly, the pioneer invasive population (East Med) started with higher Y(II) as well as the highest diatoms biomass (Chl *a*), but this did not result in better performance at colder temperatures. In fact, compared to the invasion front and source population, the East Med population seems to be the least adapted to cold stress. This suggests that the physiological performance in symbiont-bearing LBF is more tightly linked to the quality of their symbiotic associations than to the abundance of symbionts, as also found in host-symbiotic relationships in sponges (Freeman et al., 2013).

The Chl *a* analysis revealed a peculiar pattern in which the symbionts' biomasses decreased with the drop in temperature but increased in the coldest treatment at 10°C (Figure 5). This pattern is observed in all populations but is more expressed in the invasive populations. This sudden increase in symbionts biomass with cold stress is consistent with the findings of Titelboim et al. (2021) who observed that cold stress induces major reorganization of metabolic processes in invasive *A. lobifera* leading to the upregulation of genes involved in photosynthesis. Therefore, it is likely that on the course of the four-week experiment, the different populations reorganized their metabolic processes to favor photosynthesis, leading to this higher symbiont's biomass. However, only the population at the invasion front was successful in converting this effort into healthy photosynthesis (Figure 2).

In an opposite trend from the symbionts, the host/holobiont physiological responses did not differ between the different populations in the invasive range. All populations exhibited reduced growth rates with cold-temperature stress (≤ 16°C). A higher growth rate of the Red Sea population can be noticed in all treatments, but we attribute this to the smaller initial sizes of the Red Sea specimens and therefore a different growth stage. All populations also showed similar motility responses, with no pseudopodal activity at colder temperatures. The lack of pseudopodal activity alone is not an indication of stress or mortality of the host. However, the observed consistent lack of movement since Week 2 in all the 360 specimens exposed to the coldest treatments (10 and 13°C) is a strong sign of a reduction in the activity of the host. In fact, this could be a validation of the proposition by Titelboim et al. (2021) that the holobiont relocates its cellular resources to keep photosynthesis under cold stress. Either actively or just because the host is less thermally tolerant than the symbionts, the holobiont copes with cold stress by maintaining photosymbiosis with the cost of reducing growth (Titelboim et al., 2021) and movement. Moreover, the survival of the holobiont associated with a reduction in growth

and movement of the host is consistent with the concept of "dormancy", observed in different foraminifera species under stress (Ross and Hallock, 2016).

The foraminifera ability to recover from cold stress was previously observed in laboratory experiments with a sister species from the Red Sea, *Amphistegina radiata* (Zmiri et al., 1974). The authors found that the foraminifera does not move if placed in temperatures below 12°C but can recover their normal movement if returned to warmer temperatures after 4 days of being dormant (Zmiri et al., 1974). In corals, a 12h-exposure at 12°C were sufficient to disable the holobiont to recover their photosynthetic efficiency, likely due to the cold temperatures effects on the host cells rather than in the symbionts (Saxby et al., 2003). Little we know about the effects of the cold stress on the host cells: if the foraminifera host would be permanently damaged after a prolonged exposure to cold temperatures, like observed in corals, or if they would be able to recover when back in warmer temperatures after the winter has passed. It is important to highlight that, based on our observations during the experiment, the foraminifera retracts its pseudopodia when dormant and becomes a particle that is carried together with the water movement. Therefore, in nature, these dormant specimens could be easily carried by currents, which could result in a faster dispersal so far mainly attributed to the transport of juveniles (Alve and Goldstein, 2003) or passage via fish guts (Guy-Haim et al., 2017). In the scenario that the foraminifera could recover from cold stress and return its activity when back in warmer temperatures this could result in the colonization and establishment in a new location and extension of their range, currently believed to be limited by the 14°C isotherm (Triantaphyllou et al., 2012).

Cold sensitivity is known to control the range expansion of corals (Hoegh-Guldberg et al., 2005; Lirman et al., 2011; Nielsen et al., 2020; Saxby et al., 2003), and to induce bleaching in symbiotic sea anemones (Steen and Muscatine, 1987). Therefore, *A. lobifera* seems to be better adapted for range expansion compared to other marine holobionts, which explains its capacity to rapidly spread and thrive (Guastella et al., 2019; Raposo et al., submitted) in the new habitat. Even the sister-species *Amphistegina lessonii* seems to be limited by colder winter temperatures (Titelboim et al., 2019), resulting in rare records of this species in the Mediterranean (Hollaus & Hottinger, 1997). Since the invasive *A. lobifera* host shows no genetic divergence from the source population (Raposo et al., submitted), we believe this better adaptation is tightly linked to the higher diversity of symbionts in *A. lobifera* (Prazeres et al., 2021). In fact, this is the opposite pattern of what is observed in some coral species, where the range expansion is accompanied by reduced genetic diversity of the symbionts (Grupstra et al.,

2017). The wider diatoms consortia in invaded *A. lobifera* could therefore allow the holobiont to occupy a broader fundamental niche given their tolerance to warmer (Schmidt et al., 2016; Titelboim et al., 2019) and colder (Titelboim et al., 2021; present study) temperatures as compared to the ones experienced in the native habitat (Figure 6).

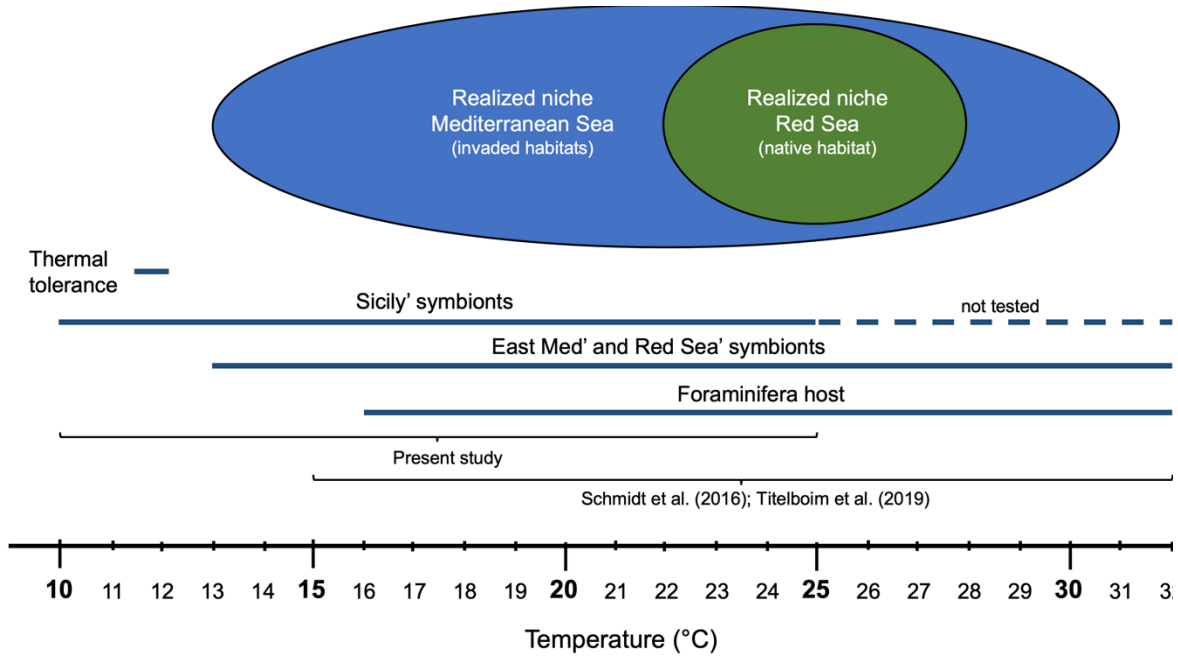


Figure 6. *Realized niches (i.e., environmental conditions experienced in nature) of native a invaded habitats and thermal tolerances (possible fundamental niches, i.e., environmental conditions they can tolerate) of Amphistegina lobifera host and symbionts*

Indeed, our findings indicate that the thermal tolerance of the host is narrower than that of the symbionts (Figure 6). The host sustains pre-adaptation to warmer temperatures (Schmidt et al., 2016; Titelboim et al., 2019) that was shaped by millions of years of evolution in the tropics (Prazeres et al., 2020), but not to colder temperatures. Therefore, the gap between the thermal tolerance of the host and the realized niche in the invaded habitat is only covered by the symbionts' physiological performance (Figure 6). Moreover, the symbionts from Sicily sustained a wider cold tolerance than the symbionts from the Levant populations (Red Sea and East Med) which is even wider than the realized niche experience in the invasive setting, where winter temperatures do not go below 13°C (Schmidt et al., 2016). This could therefore indicate that the *A. lobifera* holobiont from Sicily has the thermal adaptation needed to further spread in the Mediterranean. In conclusion, despite a lack of cold tolerance from the foraminifera host, *A. lobifera* can succeed in invasion due to their advantageous symbionts that provide the

holobiont with a wider fundamental niche. This means that even without adaptations, the niche conservatism concept can be undermined by the formation of novel biotic interactions with the invader.

Acknowledgments

We would like to thank the Israel Nature and Parks Authority for supporting both sampling campaigns, which were carried out in Israel under permission 42055/2018, as well as Dr. Miguel Frada, who graciously offered lab access at the IUI biological station. We also would like to thank Prof. Antonio Caruso for kindly advising us at the sampling campaign in Sicily. This research was supported by the BMBF-MOST collaboration in Marine Sciences Grant No. 03F0820A "ForaInva," as well as the German Research Foundation's Cluster of Excellence "The Ocean Floor—Uncharted Earth's Interface" (DFG).

Additional Information

Ethics declarations

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

All authors contributed to the writing and discussion of the study. The physiological experiment was planned together with DSR, CS, RM, and MK. Material preparation and field trips were performed by DSR, DT, SA, and RM. Data analysis was performed by DSR, CH, MK, and RM. All authors read and approved the final manuscript.

Supplementary Information

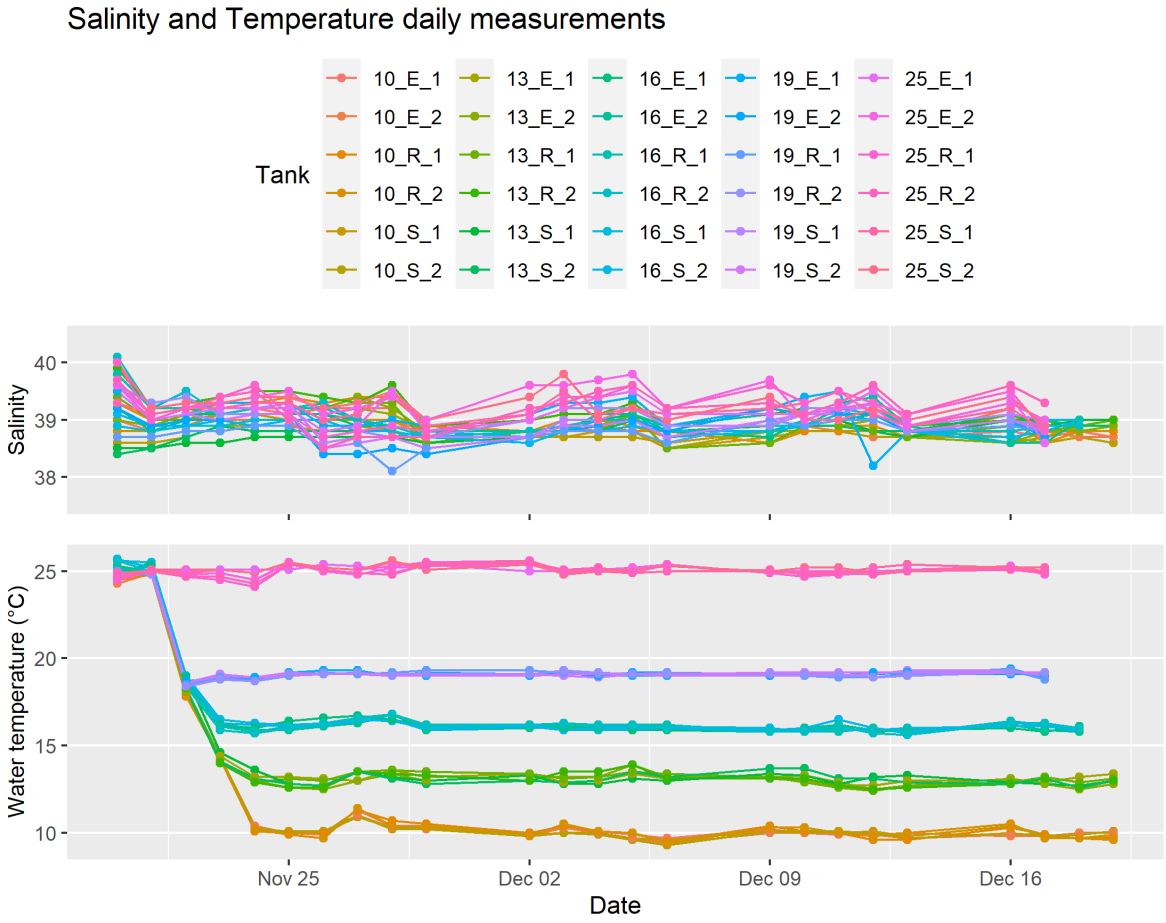


Figure S1. *Daily measurements of temperature and salinity during cold temperature experiment. Notes: The temperature was kept at 25°C until the beginning of the experiment, when it was decreased constantly until all the treatments reached the desired temperature.*

Figure S2. *Photochemistry (symbionts) responses of the entire period of experiment. "Initial" refers to the measurement before the beginning of the experiment.*

Tables only available in the digital version of this dissertation:

Table S1: Temperature records during shipment with HOBO logger (Onset, USA)

Table S2: Temperature records during physiological experiment with HOBO logger (Onset, USA)

Table S3. Analysis of variance of mixed effects logistic regression models for the binary variable (motility) to test for significance of each factor: temperature (Temp), population (Pop) and time (Week).

Notes: Z value is in reference to the Wald test and LR stands for Likelihood ratio.

Table S4: Post hoc pairwise t tests of photochemistry responses (Fv:Fm and Y(II)) for all interactions among the factor Population (Red Sea, East Med and Sicily), Time (Week 0 to Week 4) and Temperature (10 to 25°C). P value adjusted by the False Descovery Rate "FDR" method, for multiple comparisons. $P < 0.05$ represented by bold number.

Table S5: Growth measurements

Table S6: Motility measurements

Table S7: Photochemistry response of symbionts (Fv:Fm, Y(II)) measurements

Table S8: Chl *a* measurements

References

Alve, E., Goldstein, S.T., 2003. Propagule transport as a key method of dispersal in benthic foraminifera (Protista). Limnol Oceanogr 48, 2163–2170. https://doi.org/10.4319/lo.2003.48.6.2163

Bates, D., Mächler, M., Bolker, B., Walker, S., 2015. Fitting Linear Mixed-Effects Models Using lme4. J Stat Softw 67, 1–48. https://doi.org/10.18637/jss.v067.i01

Bellard, C., Cassey, P., Blackburn, T.M., 2016. Alien species as a driver of recent extinctions. Biol Lett 12. https://doi.org/10.1098/rsbl.2015.0623

Caruso, A., Cosentino, C., 2014. The first colonization of the Genus Amphistegina and other exotic benthic foraminifera of the Pelagian Islands and south-eastern Sicily (central Mediterranean Sea). Mar Micropaleontol 111, 38–52. https://doi.org/10.1016/j.marmicro.2014.05.002

el Kateb, A., Stalder, C., Stainbank, S., Fentimen, R., Spezzaferri, S., 2018. The genus Amphistegina (Benthic foraminifera): Distribution along the southern Tunisian coast. Bioinvasions Rec 7, 391–398. https://doi.org/10.3391/bir.2018.7.4.06

Freeman, C.J., Thacker, R.W., Baker, D.M., Fogel, M.L., 2013. Quality or quantity: Is nutrient transfer driven more by symbiont identity and productivity than by symbiont abundance? ISME Journal 7, 1116–1125. https://doi.org/10.1038/ismej.2013.7

Greenspan, S.E., Lyra, M.L., Migliorini, G.H., Kersch-Becker, M.F., Bletz, M.C., Lisboa, C.S., Pontes, M.R., Ribeiro, L.P., Neely, W.J., Rezende, F., Romero, G.Q., Woodhams, D.C.,

Haddad, C.F.B., Toledo, L.F., Guilherme Becker, C., 2019. Arthropod-bacteria interactions influence assembly of aquatic host microbiome and pathogen defense. Proceedings of the Royal Society B: Biological Sciences 286. https://doi.org/10.1098/rspb.2019.0924

Grupstra, C.G.B., Coma, R., Ribes, M., Leydet, K.P., Parkinson, J.E., McDonald, K., Catllà, M., Voolstra, C.R., Hellberg, M.E., Coffroth, M.A., 2017. Evidence for coral range expansion accompanied by reduced diversity of Symbiodinium genotypes. Coral Reefs 36, 981–985. https://doi.org/10.1007/s00338-017-1589-2

Guastella, R., Marchini, A., Caruso, A., Cosentino, C., Evans, J., Weinmann, A.E., Langer, M.R., Mancin, N., 2019. "Hidden invaders" conquer the Sicily Channel and knock on the door of the Western Mediterranean sea. Estuar Coast Shelf Sci 225, 106234. https://doi.org/10.1016/j.ecss.2019.05.016

Guisan, A., Thuiller, W., 2005. Predicting species distribution: offering more than simple habitat models. Ecol Lett 8, 993–1009. https://doi.org/https://doi.org/10.1111/j.1461-0248.2005.00792.x

Guy-Haim, T., Hyams-Kaphzan, O., Yeruham, E., Almogi-Labin, A., Carlton, J.T., 2017. LETTER A novel marine bioinvasion vector: Ichthyochory, live passage through fish Scientific Significance Statement. Limnol Oceanogr Lett 2. https://doi.org/10.1594/PANGAEA.872632

Hallock, P., Williams, D.E., Fisher, E.M., Toler, S.K., 2006a. Bleaching in Foraminifera with Algal Symbionts: Implications for Reef Monitoring and Risk Assessment FORAMS 2006. Anuário do Instituto de Geociências -UFRJ 29, 108–128.

Hallock, P., Williams, S.K., Toler, E.M., Fisher, Talge, H.K., 2006b. Bleaching in reef-dwelling foraminifers: implications for reef decline. 10th Int. Coral Reef Symp 737, 729–737.

Hoegh-Guldberg, O., Fine, M., Skirving, W., Johnstone, R., Dove, S., Strong, A., 2005. Coral bleaching following wintry weather. Limnol Oceanogr 50, 265–271. https://doi.org/10.4319/lo.2005.50.1.0265

Hyams, O., Almogi-Labin, A., Benjamini, C., 2002. Larger foraminifera of the southeastern Mediterranean shallow continental shelf off Israel. Israel Journal of Earth Sciences 51, 169–179. https://doi.org/10.1560/QAE1-KTEE-2K2L-QPCH

Langer, M.R., Mouanga, G.H., 2016. Invasion of amphisteginid foraminifera in the Adriatic Sea. Biol Invasions 18, 1335–1349. https://doi.org/10.1007/s10530-016-1070-0

Langer, M.R., Weinman, A.E., Lotters, S., Rodder, D., 2012. '"Strangers"' in Paradise: Modeling the Biogeographic Range. J Foraminifer Res 42, 234–244.

Lee, J.J., Anderson, O.R., 1991. Symbiosis in Foraminifera, in: Biology of Foraminifera. Academic Press, London, pp. 157–220.

Lirman, D., Schopmeyer, S., Manzello, D., Gramer, L.J., Precht, W.F., Muller-Karger, F., Banks, K., Barnes, B., Bartels, E., Bourque, A., Byrne, J., Donahue, S., Duquesnel, J., Fisher, L., Gilliam, D., Hendee, J., Johnson, M., Maxwell, K., McDevitt, E., Monty, J., Rueda, D., Ruzicka, R., Thanner, S., 2011. Severe 2010 cold-water event caused unprecedented mortality to corals of the Florida reef tract and reversed previous survivorship patterns. PloS One 6. https://doi.org/10.1371/journal.pone.0023047

Liu, C., Wolter, C., Xian, W., Jeschke, J.M., 2020. Most invasive species largely conserve their climatic niche. Proc Natl Acad Sci U S A 117, 23643–23651. https://doi.org/10.1073/pnas.2004289117

Luo, D., Ganesh, S., Koolaard, J., 2021. Predictmeans: Calculate Predicted Means for Linear Models. R package version 1.0.6.

Mack, R.N., Simberloff, D., Mark Lonsdale, W., Evans, H., Clout, M., Bazzaz, F.A., 2000. Biotic Invasions: Causes, Epidemiology, Global Consequences, and Control. Ecological Applications 10, 689–710. https://doi.org/https://doi.org/10.1890/1051-0761(2000)010[0689:BICEGC]2.0.CO;2

Meriç, E., Avşar, N., Yokeş, M.B., 2008. Some alien foraminifers along the Aegean and southwestern coasts of Turkey. Micropaleontology 54, 307–349.

Nielsen, J.J.V., Kenkel, C.D., Bourne, D.G., Despringhere, L., Mocellin, V.J.L., Bay, L.K., 2020. Physiological effects of heat and cold exposure in the common reef coral Acropora millepora. Coral Reefs 39, 259–269. https://doi.org/10.1007/s00338-019-01881-x

Pearman, P.B., Guisan, A., Broennimann, O., Randin, C.F., 2008. Niche dynamics in space and time. Trends Ecol Evol 23, 149–158. https://doi.org/10.1016/j.tree.2007.11.005

Pinheiro, J., Bates, D., R Core Team, 2022. Nlme: Linear and Nonlinear Mixed Effects Models. R package version 3.1-160.

Pinheiro, J.C., Bates, D.M., 2000. Mixed-Effects Models in S and S-PLUS. Springer, New York. https://doi.org/10.1007/b98882

Prazeres, M., Roberts, T.E., Ramadhani, S.F., Doo, S.S., Schmidt, C., Stuhr, M., Renema, W., 2021. Diversity and flexibility of algal symbiont community in globally distributed larger benthic foraminifera of the genus Amphistegina. BMC Microbiol 21, 1–16. https://doi.org/10.1186/s12866-021-02299-8

Prazeres, M., Uthicke, S., Pandolfi, J.M., 2016. Changing light levels induce photo-oxidative stress and alterations in shell density of Amphistegina lobifera (Foraminifera). Mar Ecol Prog Ser 549, 69–78. https://doi.org/10.3354/meps11698

R Core Team, 2022. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

Raposo, D., Zufall, R.A., Caruso, A., Titelboim, D., Abramovich, S., Hassenrück, C., Kucera, M., Morard, R., submitted. Invasion success in a Lessepsian symbiont-bearing foraminifera linked to high dispersal ability, preadaptation and suppression of sexual reproduction. Scientific Reports

Raposo, D.S., Hassenrück, C., Titelboim, D., Abramovich, S., Caruso, A., Kucera, M., Morard, R., in prep. Flexible symbiosis in a larger benthic foraminifera along its invasion gradient in the Mediterranean Sea.

Rilov, G., Galil, B., 2009. Marine Bioinvasions in the Mediterranean Sea – History, Distribution and Ecology 549–575. https://doi.org/10.1007/978-3-540-79236-9_31

Ross, B.J., Hallock, P., 2016. Dormancy in the foraminifera: a review. The Journal of Foraminiferal Research 46, 358–368. https://doi.org/10.2113/gsjfr.46.4.358

Saxby, T., Dennison, W.C., Hoegh-Guldberg, O., 2003. Photosynthetic responses of the coral Montipora digitata to cold temperature stress. Mar Ecol Prog Ser 248, 85–97. https://doi.org/10.3354/meps248085

Schmidt, C., Heinz, P., Kucera, M., Uthicke, S., 2011. Temperature-induced stress leads to bleaching in larger benthic foraminifera hosting endosymbiotic diatoms. Limnol Oceanogr 56, 1587–1602. https://doi.org/10.4319/lo.2011.56.5.1587

Schmidt, C., Morard, R., Prazeres, M., Barak, H., Kucera, M., 2016. Retention of high thermal tolerance in the invasive foraminifera Amphistegina lobifera from the Eastern Mediterranean and the Gulf of Aqaba. Mar Biol 163. https://doi.org/10.1007/s00227-016-2998-4

Schneider, C.A., Rasband, W.S., Eliceiri, K.W., 2012. NIH Image to ImageJ: 25 years of image analysis. Nat Methods 9, 671–675. https://doi.org/10.1038/nmeth.2089

Simberloff, D., Martin, J.L., Genovesi, P., Maris, V., Wardle, D.A., Aronson, J., Courchamp, F., Galil, B., García-Berthou, E., Pascal, M., Pyšek, P., Sousa, R., Tabacchi, E., Vilà, M., 2013. Impacts of biological invasions: What's what and the way forward. Trends Ecol Evol 28, 58–66. https://doi.org/10.1016/j.tree.2012.07.013

Sorgente, R., Drago, A.F., Ribotti, A., 2003. Seasonal variability in the Central Mediterranean Sea circulation.

Steen, R.G., Muscatine, L., 1987. LOW TEMPERATURE EVOKES RAPID EXOCYTOSIS OF SYMBIOTIC ALGAE BY A SEA ANEMONE. Biol Bull 172, 246–263. https://doi.org/10.2307/1541797

Stuhr, M., Meyer, A., Reymond, C.E., Narayan, G.R., Rieder, V., Rahnenführer, J., Kucera, M., Westphal, H., Muhando, C.A., Hallock, P., 2018. Variable thermal stress tolerance of the reef-associated symbiont-bearing foraminifera Amphistegina linked to differences in symbiont type. Coral Reefs 37, 811–824. https://doi.org/10.1007/s00338-018-1707-9

Tingley, R., Vallinoto, M., Sequeira, F., Kearney, M.R., 2014. Realized niche shift during a global biological invasion. Proc Natl Acad Sci U S A 111, 10233–10238. https://doi.org/10.1073/pnas.1405766111

Titelboim, D., Almogi-Labin, A., Herut, B., Kucera, M., Asckenazi-Polivoda, S., Abramovich, S., 2019. Thermal tolerance and range expansion of invasive foraminifera under climate changes. Sci Rep 9, 1–5. https://doi.org/10.1038/s41598-019-40944-5

Titelboim, D., Thangarjan, S., Raposo, D., Morard, R., Kucera, M., Ashckenazi-Polivoda, S., Almogi-Labin, A., Herut, B., Manda, S., Abramovich, S., Gold, D.A., Abdu, U., 2021. The transcriptomic signature of cold and heat stress in benthic foraminifera—Implications for range expansions of marine calcifiers. Funct Ecol 35, 2679–2690. https://doi.org/10.1111/1365-2435.13929

Triantaphyllou, M. v., Dimiza, M.D., Koukousioura, O., Hallock, P., 2012. Observations on the life cycle of the symbiont-bearing foraminifer Amphistegina lobifera Larsen, an invasive species in coastal ecosystems of the ilose sea (Greece, E. Mediterranean). J Foraminifer Res 42, 143–150. https://doi.org/10.2113/gsjfr.42.2.143

Wiens, J.A., Stralberg, D., Jongsomjit, D., Howell, C.A., Snyder, M.A., 2009. Niches, models, and climate change: Assessing the assumptions and uncertainties. Proc Natl Acad Sci U S A 106, 19729–19736. https://doi.org/10.1073/pnas.0901639106

Wiens, J.J., Graham, C.H., 2005. Niche Conservatism: Integrating Evolution, Ecology, and Conservation Biology. Annu Rev Ecol Evol Syst 36, 519–539. https://doi.org/10.1146/annurev.ecolsys.36.102803.095431

Yokes, M.B., Meric, E., Avsar, N., 2007. On the Presence of alien foraminifera Amphistegina lobifera Larsen on the coasts of the Maltese Islands. Aquat Invasions 2, 439–441. https://doi.org/10.3391/ai.2007.2.4.15

Zenetos, A., Gofas, S., Morri, C., Rosso, A., Violanti, D., Garcia Raso, J., Cinar, M., Almogi-Labin, A., Ates, A., Azzurro, E., Ballesteros, E., Bianchi, C., Bilecenoglu, M., Gambi, M., Giangrande, A., Gravili, C., Hyams-Kaphzan, O., Karachle, P., Katsan, M., 2012. Alien species in the Mediterranean Sea by 2012. A contribution to the application of European Union's Marine Strategy Framework Directive (MSFD). Part 2. Introduction trends and pathways. Mediterr Mar Sci 13, 328–352. https://doi.org/https://doi.org/10.12681/mms.327

Zmiri, A., Kahan, D., Hochstein, S., Reiss, Z., 1974. Phototaxis and Thermotaxis in Some Species of Amphistegina (Foraminifera). J Protozool 21, 133–138. https://doi.org/10.1111/j.1550-7408.1974.tb03626.x

Appendix Chapter

The transcriptomic signature of cold and heat stress in benthic foraminifera – Implications for range expansions of marine calcifiers

This work has been published in the Journal *Functional Ecology*, 31 Ago 2021.

Danna Titelboim[1*], Starlin Thangarjan[2], Débora Raposo[3], Raphaël Morard[3], Michal Kucera[3], Sarit Ashckenazi-Polivoda[4,5], Ahuva Almogi-Labin[6], Barak Herut[7], Sneha Manda[2], Sigal Abramovich[2], David A. Gold[8], Uri Abdu[2]

[1]School of Earth Sciences, University of Bristol, Bristol, UK

[2]Ben-Gurion University of the Negev, Beer Sheva, Israel

[3]MARUM – Center for Marine Environmental Sciences, University of Bremen, Bremen, Germany

[4]Dead Sea and Arava Science Center, Masada National Park, Mount Masada, Israel

[5]Ben-Gurion University of the Negev, Eilat Campus, Eilat, Israel

[6]Geological Survey of Israel, Jerusalem, Israel

[7]Israel Oceanographic and Limnological Research, Haifa, Israel

[8]Department of Earth & Planetary Sciences, University of California, Davis, California, USA

Data availability: The raw sequencing data and assembled transcriptome generated on this work are available on NCBI under Bioproject PRJNA762500. All code and relevant input/intermediary files are available on GitHub at https://github.com/DavidGoldLab/2021_Amphistegina_RNA-Seq

ABSTRACT

1. Global warming permits range expansions of tropical marine species into mid-latitude habitats, where they are, however, faced with cold winter temperatures. Therefore, tolerance to cold temperatures may be the key adaptation controlling zonal range expansion in tropical marine species.
2. Here we investigated the molecular and physiological response to cold and heat stress in a tropical symbiont-bearing foraminifera that has successfully invaded the Eastern Mediterranean.
3. Our physiological measurements indicate thermal tolerance of the diatom symbionts but a decrease of growth for the foraminifera host under both cold and warm stress.
4. The combined ("holobiont") transcriptome revealed an asymmetric response in short-term gene expression under cold versus warm stress. Cold stress induced major reorganization of metabolic processes, including regulation of genes involved in photosynthesis.
5. Analyses limited to genes that are inferred to belong to the symbionts confirm that the observed pattern is due to changes in the regulation of photosynthesis-related genes and not due to changes in abundance of the symbionts.
6. In contrast to cold stress, far fewer genes change expression under heat stress and those that do are primarily related to movement and cytoskeleton. This implies that under cold stress, cellular resources are allocated to the maintenance of photosynthesis, and the key to zonal range shifts of tropical species could be the cold tolerance of the symbiosis.

Keywords

Global warming, Large Benthic Foraminifera, Thermal stress, Transcriptomic signature, Cold temperature limitations , Biogeographical expansion

1. INTRODUCTION

In the face of global change, the fitness of many species in their native habitat is reduced. If not compensated by adaptation, this loss of fitness may lead to reduced competitiveness and ultimately to extinction (Root et al., 2003). At the same time, global change may create new

suitable environments outside of the native habitats of affected species, driving range expansion or shifts in species distribution (Elith & Leathwick, 2009). Under this scenario, a warming trend will force many tropical and subtropical taxa into mid-latitudes, where they should benefit over the native taxa from their physiological adaptations to higher temperatures. However, because of the shape of the Earth's orbit and the tilt of the Earth's axis, seasonality increases with latitude irrespective of global temperature. This means that as tropical invaders expand into mid-latitudes, they will experience a new thermal regime with winter temperatures far below those of the thermally buffered tropics. Therefore, unless they can migrate in pace with the seasonal cycle, the invaders need to be tolerant to both warm and cold conditions in order to thrive in their new habitat. Thus, temperature sensitivity at the cold-end of the temperature range of warm-water taxa may be the key factor limiting range expansion for tropical species in warming oceans (Vergés et al., 2014).

In the marine environment, many taxa are motileand dispersal is promoted by currents and mixing. Therefore, response to global warming by seasonally dynamic range expansion is widespread in marine taxa (Poloczanska et al., 2016). However, most subtropical and tropical shallow-water benthos, including reef-building organisms, lack the mobility to adjust their range in pace with the seasonal cycle. Thus, such taxa should be particularly vulnerable to seasonal temperature shifts. The vulnerability of reef-building organisms to temperature is associated with the prevalence of symbiotic relationships with photosynthetic algae. The symbiosis facilitates reef growth by providing metabolites and promoting calcification, but the metabolic gain of this tight relationship is traded off with higher sensitivity to perturbations (Stanley & Lipps, 2011). Under exposure to elevated temperatures, corals and other reef-building organisms lose symbionts through bleaching (Hughes et al., 2017; Lesser, 1997), or suffer from diseases (Rosenberg & Ben-Haim, 2002). In order to predict their fate under further warming, significant research effort has been directed towards the understanding of the physiological and molecular mechanisms of heat stress (Cziesielski, Schmidt-Roach, & Aranda, 2019, and references therein). But because range expansion cannot be seasonally dynamic, their success in the new habitats will also be determined by their ability to cope with cold stress. Indeed, at present time the distribution of many marine shallow-water organisms appears to be determined by minimum temperatures (Stuart-Smith, Edgar, & Bates, 2017), causing the current warming process to control the edge of their expansion range. Remarkably, until now, very little research has been done on the mechanisms of response to cold stress in marine calcifying organisms. In theory, the effect of temperature stress on metabolic processes

should be asymmetrical because higher temperature leads to faster reaction kinetics, implying that adaptations to cold and heat stress may require different types of molecular responses.

A valuable model to examine mechanisms of thermal sensitivity in invasive reef-builders are symbiont-bearing Large Benthic Foraminifera (LBF). These single-celled organisms are prolific calcifiers and important ecosystem engineers in warm-water coastal settings. Similar to corals, they inhabit tropical-subtropical waters where they can populate a variety of substrates (Kitazato, 1988; Murray, 2006; Reiss & Hottinger, 1984). Similar to other eukaryotic microbes and marine invertebrates, their key method of range expansion is propagule transport (Alve & Goldstein, 2003), while the motility of adult individuals is limited (Dupuy, Rossignol, Geslin, & Pascal, 2010; Murray, 2006). Temperature is a major factor controlling the distribution of LBF, and different species are known to exhibit specific thermal thresholds for reproduction, survival, bleaching, and calcification (Langer & Hottinger, 2000). Unlike corals, LBF host diverse consortia of algal symbionts (Prazeres & Renema, 2019) and it is likely that thermal adaptations of the symbionts may explain the range and variability of Species-specific thermal tolerance (Pinko, Abramovich, & Titelboim, 2020; Schmidt, Morard, Romero, & Kucera, 2018; Stuhr, Meyer, et al., 2018). The small size and high abundance of LBF make it possible to obtain large samples for field and laboratory studies, allowing sufficient statistical power to test the observed patterns with negligible impact on the sampled ecosystem (Hallock, Lidz, Cockey-Burkhard, & Donnelly, 2003). Alongside physiological experiments, LBF are increasingly amenable to the study of molecular mechanisms of stress resistance by quantification of protein composition (Doo et al., 2012; Stuhr, Blank-Landeshammer, et al., 2018). Here we focus on the species *Amphistegina lobifera*, an extremely abundant calcifier that has successfully invaded the Eastern Mediterranean and is presently found in great numbers along the Israeli Mediterranean coast (Hyams-Kaphzan, L., & Almogi-Labin, 2014). Its invasion seems to be linked to the ability of this species to occupy a broad thermal niche that allows it to withstand the seasonal temperature range throughout the Eastern Mediterranean (Titelboim et al., 2019).

Here we take advantage of this model system and Investigate the molecular response of *A. Lobifera* and its diatom symbionts to cold and heat stress. To this end, we determine the transcriptomic signature in an experiment where the physiological response to stress of the exposed populations has been monitored. Whereas physiological monitoring can only record the loss of fitness due to stress, and is expected to be symmetrical (loss of performance under

cold and warm conditions), the potentially asymmetrical molecular-level response can be deciphered by documenting the differential gene expressions.

2. MATERIALS AND METHODS

All code used in this paper along with relevant intermediate files can be accessed at GitHub: https://github.com/DavidGoldLab/2021_Amphistegina_RNA-Seq

2.1 Study Design

Three sets of temperature manipulation experiments were performed in this study. The first (transcriptome experiment) combined the study of differential gene expression with the monitoring of the overall performance (growth) of the holobiont by quantification of its calcification rates. For this experiment, specimens were collected in July 2018 during summer when the average ambient temperature was 29ºC. The specimens were kept in the lab for 3 weeks prior to the experiment. The second (physiological experiment) separately examined the overall holobiont performance and the performance of the diatom symbionts by measuring changes in calcification rates (holobiont growth) and net photosynthesis (performed by the symbiont diatom algae). Specimens were collected in December 2019 when the ambient average temperature was 17ºC. The specimens were kept in the lab for 3 weeks prior to the experiment. The third experiment (photophysiology responses experiment), aimed to quantify the photosynthetic efficiency of the diatom symbionts, focusing on the lower temperature range, in order to ratify the observation regarding the effect of temperatures on photosynthesis. Specimens were collected in October 2019 and ambient temperature was 26ºC. The specimens were kept in the lab for ~4 weeks prior to the experiment. Each experiment spanned three weeks.

2.2 Culturing *Amphistegina lobifera*

Samples were collected from the rock flats at Tel Shikmona (Northern Israel, Mediterranean) before each experiment. In the laboratory, specimens of *A. lobifera* were identified as living by brownish color given by algal symbionts and by the motion of the specimens. Only *A. lobifera* specimens larger than 0.5 mm were selected for the experiments, cleaned by brushing, and randomly divided into groups of 25 (transcriptome experiment) or 30 (physiological

experiment) individuals placed in 60 mL airtight flasks filled with unfiltered seawater for the transcriptome experiment and filtered to 0.45 µm for the physiological experiments. In the photophysiology experiment, specimens were divided into groups of 5 and placed in 15 ml flasks with artificial seawater at 38.5‰ salinity (Tropic Marin® Sea Salt Classic). These groups of specimens kept in the same flask are henceforth referred to as 'samples'. Each sample contained individuals randomly chosen from the population in Tel Shikmona (Figure 1).

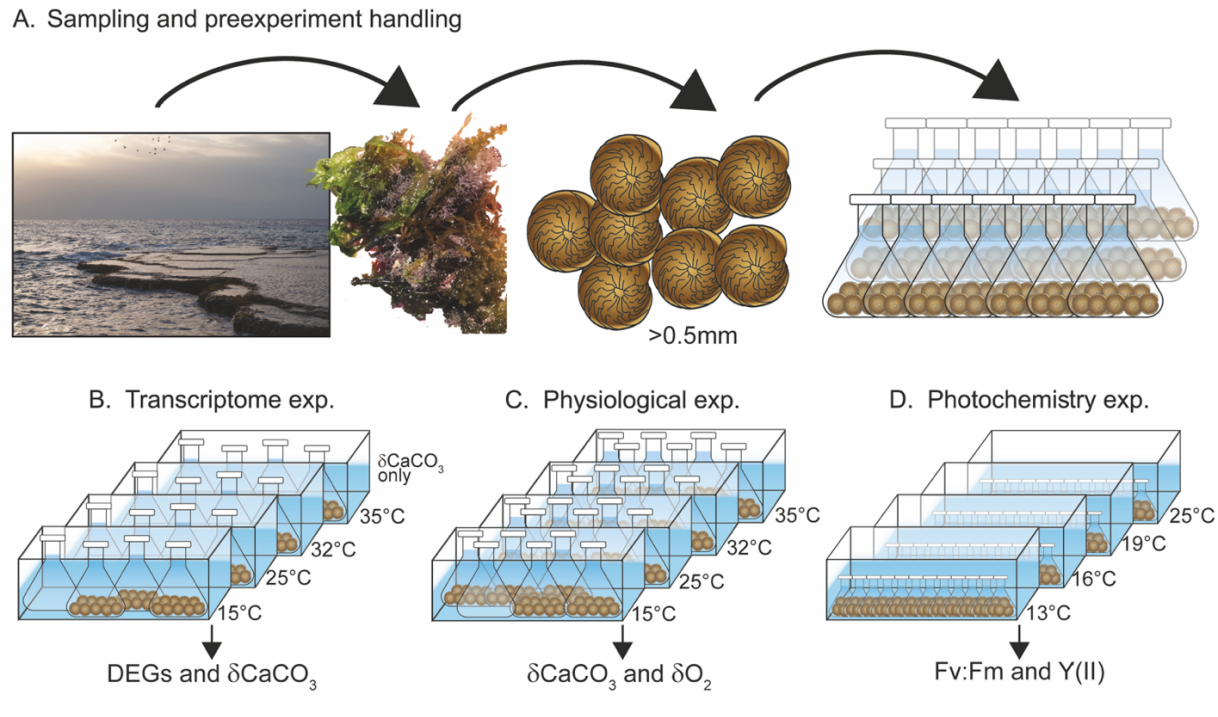


Figure 1. *Experimental design: A: sampling and preparation of the samples, B – D: experimental design for each of the experiments and their products including differentially expressed genes (DEGs), calcification rates (CaCO3), net photosynthesis (dO2) and efficiency of the photosystem II (Fv:Fm and Y(II)).*

For all experiments, after the lab acclimation period at 25°C, a ramp-up/down process of 0.2 – 1ºC per hour was performed until the designated temperature has been reached. The physiological experiment included four temperature treatments: 15ºC, 25ºC, 32ºC, 35ºC, with five replicates for each treatment. The temperature treatments were chosen to represent non-lethal thermal stress and also include optimal temperature conditions (25ºC) for *A. lobifera* (Schmidt, Morard, Prazeres, Barak, & Kucera, 2016; Titelboim et al., 2019). Moreover, the treatments cover the range this species is naturally exposed to in the Eastern Mediterranean (15-31ºC) and also include treatments of future warming scenarios (32ºC, 35ºC). Holobiont growth (calcification rates) was determined using the Alkalinity anomaly method (Smith & Key, 1975), and photosynthesis was determined by oxygen concentration compared to a control

(i.e., a bottle with no foraminifera exposed to the same conditions as the other bottles). Dissolved oxygen was measured using Eutech DO 450 connected to a rugged dissolved oxygen (RDO) sensor. A detailed description of the methods can be found in (Pinko et al., 2020; Titelboim et al., 2019). Specimens for the transcriptome experiment were exposed for three weeks to three temperatures, 15ºC, 25ºC, 32ºC, including three replicate samples for each treatment. The calcification rates of these samples in each temperature were measured at the end of the experiment. This measurement was taken for additional acute treatment (that did not undergo transcriptome analyses) to create a more extensive baseline for comparison between experiments (Figure 1).

The photophysiology experiment focused on the lower temperature range and included four treatments (13ºC, 16ºC, 19ºC, 25ºC) with 12 replicates per treatment. The efficiency of the photosystem II (PSII) was investigated by Pulse Amplitude Modulated (PAM) fluorometry, using an IMAGING-PAM Fluorometer (M-Series, WALZ GmbH, Germany), with the "MAXI-Head,1/2" CCD camera equipped with LED lights and the zoom objective (F1.0/f = 8–48 mm). For every sample, the five specimens were measured together. After 15-30 min of dark adaptation, the maximum quantum yield (Fv:Fm) was measured. Next, the effective quantum yield was measured (light adapted, Y(II)) which is obtained after the exposition to a light beam at 20 µmol photons $m^{-2}s^{-1}$ (similar to the experimental light conditions) for 30 seconds duration (a detailed description of the method can be found in Schmidt et al., (2016).

The temperatures in the transcriptome and physiological experiments were manipulated in a water baths with flasks submerged in them. The temperature was monitored and did not exceed +/- 0.1ºC from the target temperature of each treatment. All flasks were kept under a 12 h light–12 h dark cycle using ~ 40 µmol photons m s of white fluorescent light.

The photophysiology experiment was conducted in an incubator and temperature did not exceed +/- 0.5ºC from the target temperatures of each treatment. Lighting was provided by lamps inside the incubators (Philips TL Mini 8W/840) programmed in a 12 h light-12 h dark cycle using ~ 25 µmol photons m s of white fluorescent light. Oxygenation was provided by pumps programmed to switch on twice a day for 30 min. To avoid a salinity increase, every week, half of the tank volume was exchanged by new artificial seawater at the same temperature of the treatment. The specimens were fed weekly with autoclaved algae *Nannochloropsis* food mixture (Schmidt et al., 2016) by adding 30 µL of the food mixture in the individual flasks.

From here on, the treatments from all experiments are referred to as acute low (13ºC), low (15/16ºC), medium (19ºC), control (25ºC), high (32ºC), and acute high (35ºC, Figure 1). At the end of all experiments, all specimens were alive, as indicated by adhesion to the culturing flask glass, indicating active pseudopodia, and no bleaching (loss of color suggesting loss of symbionts or symbiont pigments) was observed. The only exception is in the 13ºC treatment in the photophysiological response experiment, where specimens did not show signs of pseudopodial activity but were not bleached and thus considered alive

2.3 Transcriptome library construction

Total RNA was extracted using a Plant/Fungi purification Kit (Thorold, Canada) according to the manufacturer's protocol. The concentration and quality of each RNA extraction were validated using a NanoDrop 2000 (Thermo, CA, USA), and an RNA Nano 6000 Assay Kit on the Agilent Bioanalyzer 2100 system (Agilent, CA, USA). The Bioanalyzer results confirmed that all RNA Integrity Number (RIN) values were above 7.0. RNA samples were then processed by Technion Genome Center (Israel) according to the Illumina TruSeq RNA Library Preparation Kit v2. The kit uses Oligo-dT beads to capture polyA tails, meaning the mRNA from eukaryotes like foraminifera and diatoms should be captured while microbial RNA is minimized. The constructed libraries were sequenced in a single lane on program 50SR SBS V reagent, in an Illumina HiSeq 2500 System. The single-end read sequences were obtained as individual FASTQ files.

2.4 De novo transcriptome assembly

Before assembly, raw reads were trimmed by removing adaptor sequences and ambiguous nucleotides. Reads at low-quality score and length below 36 bp were filtered out using Trim-Galore v0.4.5 and cutadapt v1.15 in the NeatSeq-Flow module. The reads from all datasets were combined for de novo assembly with Trinity (Henschel et al., 2012). We tried two different approaches for transcriptome assembly. In the first approach, all cleaned reads were combined into a single "holobiont" Trinity run. In the second approach, we used the NeatSeq-Flow pipeline to compare the cleaned reads against databases of known foraminiferal and algal sequences (details provided in table S1). Reads were independently mapped to the foraminiferal and algal databases, which were then used in two separate Trinity runs were performed to generate "foraminifera" and "symbiont" specific transcriptomes. All three transcriptomes are available on GitHub.

2.5 Gene annotation

Annotation of the gene models was performed using the Trinotate pipeline (Bryant et al., 2017). Protein translations of gene models were predicted using the Transdecoder software packaged with Trinity. Gene assignments were performed using BLAST 2.9.0+ (Camacho et al., 2009). BLASTp was used for protein queries, while BLASTx was used for nucleotide queries. Both query sets were compared against the Uniprot SwissProt database of reference proteins (https://www.uniprot.org/downloads). Conserved domains were identified in the protein models using HMMER v.3.3 (http://hmmer.org) and the Pfam-A database (ftp://ftp.ebi.ac.uk/pub/databases/Pfam/current_release/Pfam-A.hmm.gz). The results were loaded into Trinotate's SQL database. Trinotate then used the top BLASTp hits to populate the database with gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) terms. The results of this annotation are available on GitHub (for the holobiont see file Trinotate.Holobiont.report.txt; for the symbiont see file Symbiont.Trinotate.report.txt)

2.6 Read mapping and differential gene expression

Transcript quantification was performed using RSEM (Li & Dewey, 2011), using Bowtie2 (Langmead & Salzberg, 2012) to map reads to the gene models. Mapping statistics were sufficient for differential gene expression; the number of reads aligned to the holobiont transcriptome ranged from ~76-78%, providing ~14-16.5 million reads per replicate (see Table S2 for details). The count matrices produced by RSEM were compiled into a single dataset using the abundance_estimates_to_matrix.pl Perl script included with Trinity. We then performed differential expression analysis using the run_DE_analysis.pl script, using DeSeq2 as our expression analysis tool to perform independent contrasts between the conditions (Love, Huber, & Anders, 2014). The R scripts used to run DeSeq2 are provided on GitHub. A principal component analysis and correlation matrix were produced to examine the similarity between biological replicates in our RNA-Seq data (Figure 2).

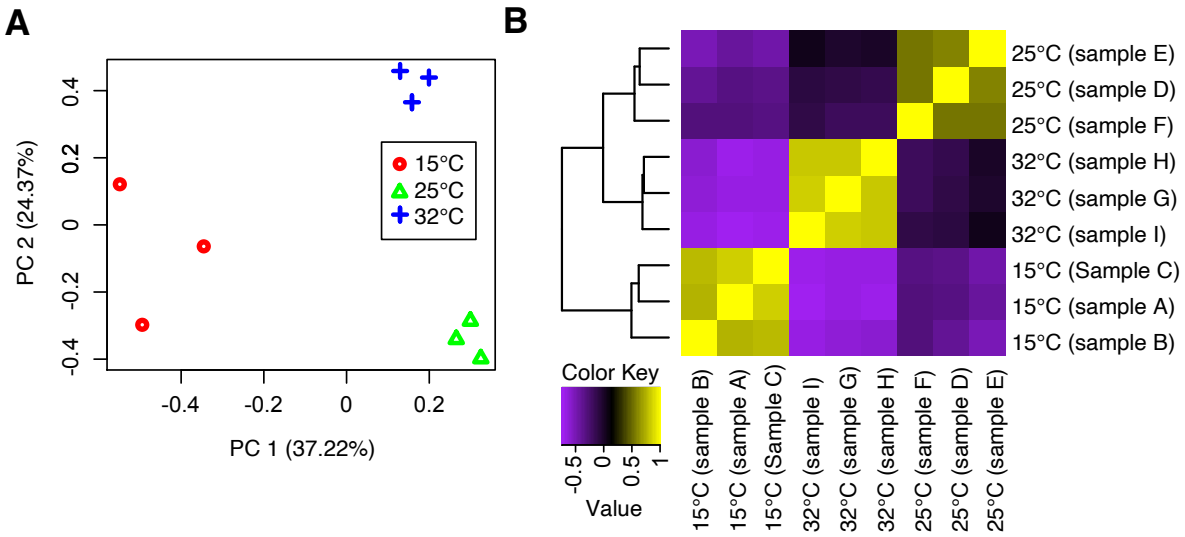


Figure 2. *Evaluating the similarity of biological replicates within and between treatments. These results apply to the holobiont transcriptome. (A) Principal component analysis based on gene counts, normalized into gene counts per million and log transformed. (B) Correlation matrix produced from the same data. This demonstrates that biological replicates are more highly correlated within treatments than between them.*

2.7 Enrichment Analyses

Enrichment analyses were performed using the GoSeq program packaged with Trinity. GO analyses were performed as described on the Trinity website. We also used GOSeq to look for enriched KEGG pathways, as GOSeq is a flexible program that allows for term sets other than GO terms to be analyzed. Trinotate provides KEGG Orthology (KO) "entry" identifiers for each annotated gene, but does not provide information on the KO "pathway" identifiers associated with that entry. We therefore used a custom script built on GNU Wget to extract this information from the KEGG website (see GitHub file: 3_DESeq2/0_Commands.txt). The data was formatted into a text document used as an alternative for GO terms in GoSeq analysis (GitHub file: 3_DESeq2/129il_annotations.txt).

Additional enrichment analyses were carried out using the GO_MWU package in R (Wright, Aglyamova, Meyer, & Matz, 2015). The output from the GoSeq analysis was used to create the "table of GO annotations" input file, and the log-fold changes calculated from DESeq2 were used to create the "table of measure of interest" input file. The commands and R scripts executed are available on GitHub (subfolder "4_GO_MWU")

2.8 Statistical analyses of the physiology experiment

To choose the appropriate statistical test for examining the differences in calcification rates, net photosynthesis, and photophysiological responses, we conducted Shapiro–Wilk test of normality and Levene's test for homogeneity of variances. The results of the net photosynthesis and effective quantum yield measurements exhibited normality and homogeneity. Thus, the significance of the differences between treatments was tested using one-way ANOVA followed by Tukey's Honestly Significant Difference (HSD) test. The measured calcification rates and maximum quantum yield violated the assumptions, and thus the differences were examined using a pairwise t-test with p values corrected according to the Benjamini-Hochberg method.

3. RESULTS

3.1 Physiological experiment

Calcification rates, which serve as a proxy for the well-being of the holobiont, were significantly lower under both heat and cold-induced thermal stress with optimum performance at 25ºC (Figure 3). The parabolic response to temperatures between 15 and 35ºC indicates that the range of temperature is wide enough to represent the thermal tolerance of the holobiont (Castillo, Ries, Bruno, & Westfield, 2014). Despite the reduction in calcification, net photosynthesis was maintained between 15ºC and 32ºC. At 35ºC, net photosynthesis decreased to negative values (-0.3±0.15), indicating that respiration exceeded oxygen production. These results suggest that calcification, which is performed by the foraminifera, diminishes under thermal stress while photosynthesis, which is performed by the symbiont, is maintained under a wider thermal range.

3.2 Transcriptome experiment

We conducted transcriptome analysis to determine how gene expression correlates with physiology. To this end, we analyzed specimens for RNA sequencing (RNA-Seq) following three weeks of exposure to temperature treatments representing non-lethal cold stress (15ºC), optimum (25ºC), and non-lethal heat stress (32ºC).

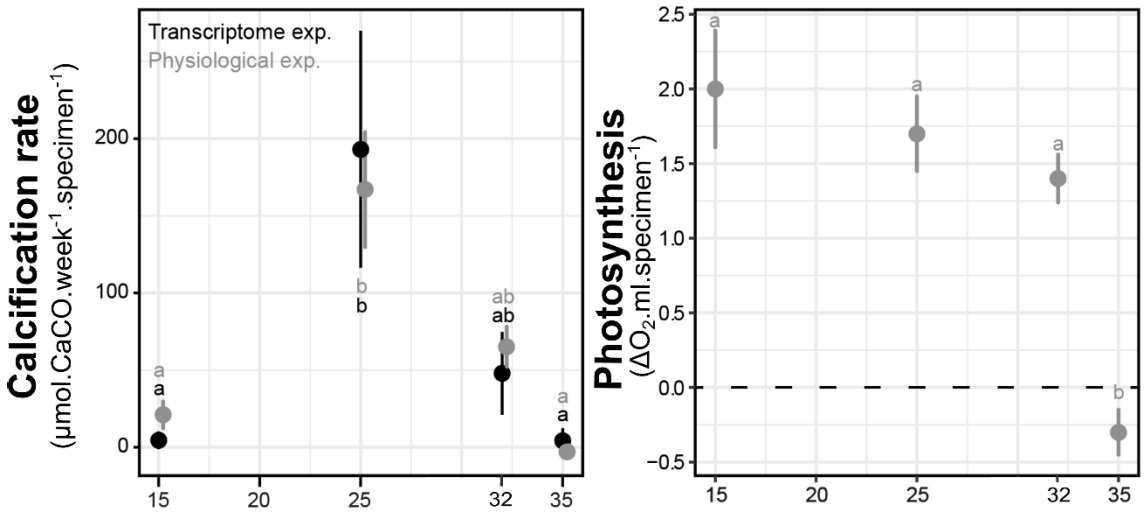


Figure 3. *Calcification rate of A. lobifera and net photosynthesis response to different temperatures. Calcification rate (left) and net photosynthesis (right), after three weeks of exposure to different temperatures. Calcification data in black represents the results when the experiment was repeated for transcriptome analysis. The letters in each box represent statistical significance ($p < 0.05$) between treatments.*

We began by investigating if we could differentiate between *A. lobifera* and symbiont transcripts. To determine this, we compared two different assembly methods, one where all raw RNA-Seq data was used in transcriptome assembly (the "holobiont" dataset), and a second where RNA-Seq reads were taxonomically assigned prior to transcriptome assembly (generating "foraminifera" and "symbiont" datasets). BUSCO analysis, which uses a set of near-universal single-copy orthologs to calculate transcriptome completeness (Seppey, Manni, & Zdobnov, 2019), gives the holobiont transcriptome a score of ~68%, slightly better than the results for *Reticulomyxa 131ilose* (~66%), the only foraminifera with a partly annotated genome (Table 1). The two datasets also produced far fewer genes than would be expected based on related organisms; the 10,087 genes in our "foraminifera" transcriptome are far fewer than what is estimated in the foraminifera *R. 131ilose* (~40,000 genes), while the 1,837 genes in our "symbiont" transcriptome pale in comparison to the diatoms *Phaeodactylum tricornutum* (~10,000 genes) and *Thalassiosira oceanica* (~31,000 genes) (Bowler et al., 2008; Glöckner et al., 2014; Lommer et al., 2012). Consistent with the low gene counts, the "foraminifera" and "symbiont" assemblies had poorer BUSCO scores than the holobiont (42.4% and 7.8%, respectively) However, the "foraminifera" and "symbiont" transcriptomes had large differences in CG content (34.13% and 50.28% respectively), consistent with values from

published genomes, which range from ~35-42% for foraminifera (Glöckner et al., 2014; Habura, Hou, Reilly, & Bowser, 2011) and ~47%-53% for diatoms (Bowler et al., 2008; Lommer et al., 2012). These results suggest that trying to taxonomically assign the RNA-Seq reads prior to transcriptome assembly was successful but lead to a significant loss of genes for both datasets, most likely because of the limited number of foraminifera and diatom genes currently available (Table S1). Given the limited number of genes that can be specifically assigned to the foraminifera or the symbiont, and since the two functions as a single unit, we decided to focus our initial analyses on the collective "holobiont".

Table 1. Summary statistics of the assembled transcriptomes. The "holobiont" represents the inclusion of all RNA reads in the Trinity assembly, while the "symbiont only" and "*A. lobifera* only" represent datasets where the reads were assigned as algal or foraminiferal sequences, respectively, prior to assembly. Contig statistics are based on the longest isoform for each gene. BUSCO completeness is calculated by combining the percent of complete and fragmented genes. Detailed analyses are available on GitHub.

	Holobiont	**symbiont only**	***A. lobifera* only**
Total number of genes	62,014	1,837	10,087
Total number of transcripts	95,266	2,005	11,855
% GC	34.86	50.28	34.13
Contig N50	732	336	335
Median contig length	408	279	278
Genes with protein translations	52,993	754	4,228
Genes with complete protein translations	3,276	16	41
BUSCO transcriptome completeness (%)	68.2	7.8	42.4

To understand the transcriptional response of the *A. lobifera* holobiont to the different temperature treatments, we applied differential gene expression to our RNA-Seq data. Using DeSeq2 (Love et al., 2014), we identified 6,182 differentially expressed gene models (defined as p-value < 0.001; log (fold change) > 2 in one or more comparisons). The number of

differentially expressed genes is asymmetrical, with more than three times as many genes exhibiting differential expression under cold stress (15ºC vs. 25ºC; 3,618 genes) compared to heat stress (25ºC vs. 32ºC; 1,112 genes). To interpret these shifts in gene expression, we tested for enriched gene ontology (GO) terms. We compared two different techniques for finding enriched GO terms: (1) the goseq R package packaged with Trinity (Young, Wakefield, Smyth, & Oshlack, 2010) and the GO_MWU R package (Wright et al., 2015). The goseq method takes a list of genes along with their gene ontology annotations, and compares it to a "background" gene list to identify enriched GO terms. The GO_MWU package uses a Mann-Whitney U test to determine whether genes belonging to a specific GO category are significantly concentrated near the top or the bottom of a global ranked list of genes (Supek, Bošnjak, Škunca, & Šmuc, 2011). Consistent with the larger number of differentially expressed genes, both methods recovered far more GO terms with lower p-values during cold stress compared to heat stress. In the goseq analysis, enriched biological processes during cold stress (295 GO terms total) are dominated by metabolism, while heat stress response (5 GO terms total) is dominated by microtubule and ciliary processes (see Table S3 for summary results and the GitHub repository for full results). These results collectively demonstrate a marked asymmetry in gene regulation, where adapting to cold stress requires more changes in gene expression and more shifts in metabolic processes compared to heat stress.

The GO_MWU analysis was consistent with results from goseq but revealed more details in the nature of the photosynthesis response during cold stress. The output of GO_MWU (Supplementary Figure 1) suggests that genes related to photosynthesis (GO:0015979) and chlorophyll metabolism (GO:0015994) are upregulated during cold stress, while the photosynthesis light-harvesting process (GO:0009765) is downregulated. Photosynthesis and chlorophyll metabolism also show up as significantly enriched GO terms during cold stress in our goseq analysis (false-discovery rate adjusted p-values = 0.005, 0.004 respectively, Kanehisa & Goto, 2000).

We were particularly interested in the recovery of photosynthesis from the RNA-Seq data, as we anticipated this process being impacted by cold stress but failed to see it in our physiological experiment. This raised the question as to whether the GO enrichment in our RNA-Seq data represented real shifts in gene expression from the photosynthetic symbionts or was a consequence of changes in symbiont abundance. To test this hypothesis, we re-ran our RNA-Seq analyses on the "symbiont" specific transcriptome. The results of this analysis must be

interpreted with caution since, as described earlier, the 1,837 genes in our symbiont "transcriptome" almost certainly represent a fraction of the true number of genes. However, it is worth noting that nearly all enriched GO terms in this dataset are related to photosynthesis under cold stress (see Table S4). This suggests that the shift in photosynthesis-related genes represents actual changes in symbiont gene expression, and not only changes in symbiont abundance. Figure 4 provides a heat map for all differentially expressed genes associated with photosynthesis. Most genes are upregulated at 15ºC, with a small cluster of FCP (fucoxanthin-chlorophyll a-c binding protein) and LHCSR (light-harvesting complex stress-related protein) related genes that are upregulated at 32ºC. These genes are part of the light-harvesting complex, which is consistent with the results produced by GO_MWU. The gene expression data ultimately lead to a counterintuitive result; although there is no significant change in oxygen production, under cold-stress, the genes responsible for photosynthesis were largely upregulated.

3.3 Photo-physiological experiment

Following the results of our gene expression study, we performed an additional analysis to look specifically at the impact of cold temperature stress on photosynthesis. The study was similar in design to the physiological experiment, except more temperatures were tested in the cold range (13ºC, 16ºC, 19ºC, and 25ºC), but instead of oxygen production, we measured the maximum quantum yield (Fv:Fm) and effective quantum yield (Y(II)) of photosynthesis (Figure 5). Consistent with our physiological study, maximum quantum yield did not show significant differences between 16ºC – 25ºC, although it did drop significantly at 13ºC. Conversely, the effective quantum yield showed a significant increase between 25ºC to 19ºC, followed by a significant decrease at 16ºC and another at 13ºC. These results suggest that symbiont photosynthesis shows a broad range of tolerance at cold temperatures, but that temperatures of 16ºC and lower negatively impact photosynthetic quantum yield and thus its efficiency.

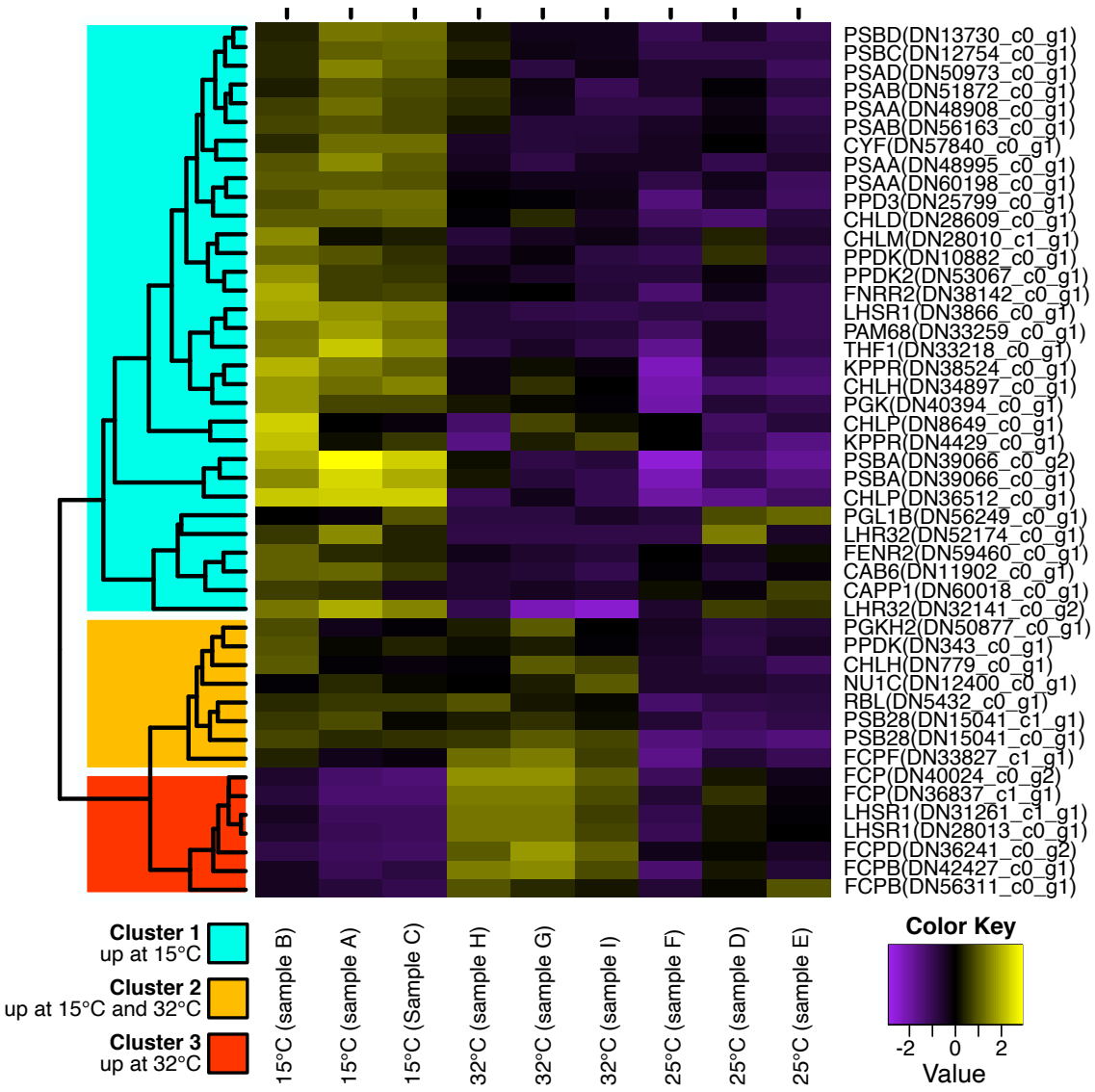


Figure 4. *Heat map of differentially expressed holobiont genes associated with photosynthesis. For this figure differential expression means genes with logfold change > 2 and a false discovery rate adjusted p-value < 0.05. Genes that met this threshold we extracted if their GO ontologies included one or more of the following annotations: photosynthesis (GO:0015979); photosynthesis, light harvesting (GO:0009765); photosynthesis, dark reaction (GO:0019685); photosynthesis, light reaction (GO:0019684); photosystem (GO:0009521). Names to the right of the heat map provide IDs for each row, including UniProt-based gene names (based on Trinotate best BLAST hits) followed by Trinity gene identifiers. The hierarchical clustering of gene expression profiles on the left suggests the genes can be divided into three distinct patterns, with the largest profile being genes that are upregulated at 15°C.*

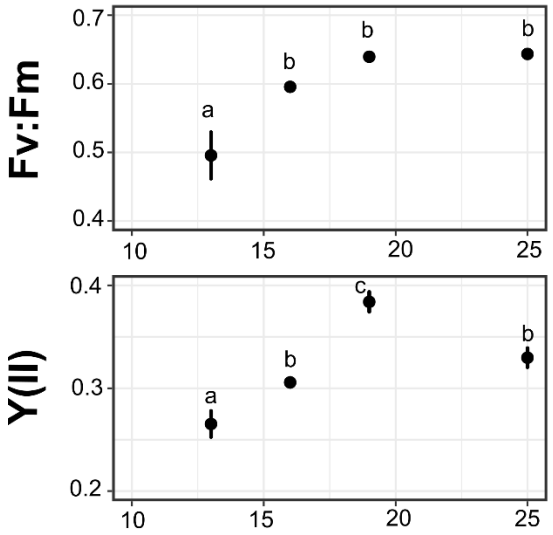


Figure 5. *Photophysiology response of the symbiont photosynthesis to different temperatures. (Top) maximum quantum yield, or Fv:Fm. (Bottom) Effective quantum yield, or Y(II). The letters in each box represent statistical significance ($p < 0.05$) between treatments.*

4. DISCUSSION

Whereas the response to heat stress has been extensively investigated in tropical marine organisms, their tolerance and adaptability to low temperatures are considerably understudied. This is despite the fact that species distributions are changing under warming, which acts to remove temperature barriers for expansion into regions with significantly higher seasonality, thus increasing exposure of tropical species to cold conditions (Root et al., 2003; Vergés et al., 2014). In this respect, the Lessepsian invasion of tropical species into the Mediterranean provides an ideal opportunity to study range expansion of marine species into an environment with elevated seasonal temperature contrast.

Previous work on Lessepsian invaders, including *Amphistegina lobifera* , in the Mediterranean, confirmed their tropical Indo-Pacific origin (Zenetos et al., 2010) and specifical populations in the Gulf of Aqaba (Schmidt et al., 2016), characterized by warm temperatures throughout the year (>28 ºC, De Deckker, 2016). Thus, their invasion into the Eastern Mediterranean confronts these organisms with an unfamiliar extreme cold winter temperature (~16 ºC, Gertman & Hecht, 2002). Among the invaders, *A. lobifera* is known to modify its geographic range in response to ongoing global warming without genetic differentiation (Prazeres et al., 2020), which explains the wide distribution in their extant populations and during historical events of climate perturbation (Langer & Hottinger, 2000). The ability of this species to expand and prosper in the Mediterranean regardless of the cold winters distinguishes it from other tropical organisms that have not invaded, and allows us to study the mechanism of cold stress

resistance. Even the closely related species *Amphistegina lessonii* is limited by the cold winter temperatures indicated by the very few reports of this species in the eastern Mediterranean and only from deeper settings (Hollaus & Hottinger, 1997), highlighting the control of temperature on species distribution (Titelboim et al., 2019).

Calcification rate and photosynthesis are proxies for the well-being of the foraminifera and their symbiont algae, respectively. Thus, our physiological measurements allowed us to evaluate certain aspects in the responses of *A. lobifera* and its diatom symbionts to the different thermal conditions. Calcification rates show optimum performance at 25ºC, whereas stress-related inhibition occurs at 15ºC and 35ºC. A similar parabolic calcification response was observed under roughly the same temperatures in the resilient reef-building coral *Siderastrea siderea* (Castillo et al., 2014; Fabry, 2008; Ries, Cohen, & McCorkle, 2009) indicating this might be the sensitivity of many tropical calcifying organisms and that calcification by reef organisms will be severely reduced with even a mild warming. On the other hand, symbiont performance in *A. lobifera* was sustained between 15-32ºC, and only inhibited at 35ºC and 13ºC, showing a wider tolerance range compared to the calcification response. The parabolic calcification response of *A. lobifera* indicates that the present-day annual temperature range in the Eastern Mediterranean offers only a narrow seasonal window with optimum conditions, indicating that the species will benefit from further warming that will shorten the time in a year in which it experiences cold stress. However, the symbionts exhibit a wider optimum range than the present-day annual temperatures, indicating they will sustain their functionality with further warming and that they will not hinder further expansion into regions where winter temperatures are cold. Moreover, the parabolic response of the holobiont is symmetrical compared to the symbiont that seems to decrease its function more sharply at the warm compared to the cold end, possibly providing the holobiont the ability to survive the cold winter in the Eastern Mediterranean in a state where it reduces growth and calcification.

Our transcriptome analyses unveiled some of the key processes that facilitate tolerance to thermal stress in *A. lobifera* and its symbiont. Comparison of the gene response under the cold and warm stress revealed an asymmetrical pattern with more DEGs in the cold treatment. This suggests that a larger reorganization of the transcriptome is needed to adjust to cold stress and that the involved processes are fundamentally different from those involved in coping with heat stress. In the high temperature (32ºC) treatment, most DEGs were related to microtubule and ciliary processes. A similar response was also observed by (Stuhr, Blank-Landeshammer, et

al., 2018), where chronic heat exposure led to a strong increase in foraminifera-associated proteins responsible for microtubule-based movement. Microtubules and related motor proteins in foraminifera are highly abundant in pseudopodia, which facilitate movement and heterotrophic food intake (Travis, Kenealy, & Allen, 1983). This would indicate that when exposed to heat stress, the holobiont is unable to maintain its photosymbiotic relationship and the foraminifera switch to increased heterotrophy.

In contrast, in the cold temperature (15ºC) treatment, the DEGs were dominated by genes coding for metabolic processes, and the most interesting observation was the change in the regulation of genes involved in photosynthesis under the low temperature treatment. This is unanticipated considering our physiological experiments that show similar net oxygen production in the low (15ºC), control (25ºC) and high (32ºC) treatments, as well as similar photo physiological response at low (16ºC) and control (25ºC), that only reduces under acute low temperature (13ºC). This suggests that under cold stress, the symbiont algae compensate the lower photosynthesis rate due to lower temperature by regulating photosynthesis genes. As a result, the holobiont appears to maintain normal levels of oxygen production, but the reallocation of cellular resources to photosynthesis genes seems to occur on the cost of holobiont growth. The observation of survival without growth is consistent with the concept of "dormancy", observed among different species of foraminifera under various stress conditions (Ross & Hallock, 2016). In our case, the "dormancy" is only manifested by lack of growth, whilst metabolic activity is maintained.

Either the host, which facilitates the overall growth, is deprived of metabolites otherwise delivered by the symbionts, or it is less thermally tolerant than the symbionts. Either way, the combined physiology and transcriptome analysis indicates that the holobiont copes with cold stress by maintaining functionality without growth, whereas during heat stress, the impairment of both growth and functionality of the photosymbiosis are known to occur.

In the Eastern Mediterranean, *A. lobifera* is naturally exposed to 15ºC in winter, whilst the tested heat stress temperature of 32ºC is slightly higher than the current summer temperatures in this region (Gertman & Hecht, 2002). This implies that the invasive *A. lobifera* is preadapted to warm temperature, an adaptation that was shaped by millions of years of evolution in the tropics (Prazeres et al., 2020) and is not lost during invasion (Schmidt et al., 2016). In contrast, the origin of the observed tolerance to cold temperatures is enigmatic. It is unlikely to provide any advantage in the tropics (not even in the Northern Red Sea from where the invasive

population is likely sourced), where such cold temperatures are never realized. A possible clue to the origin of the overall cold tolerance could be seen in the inference that the symbionts appear to be coping with the cold better than the host. *Amphistegina lobifera* is known to be flexible with regard to symbiont choice (Prazeres et al., 2020) and since foraminifera are able to obtain new symbionts from the environment, such as during sexual reproduction (Prazeres & Renema, 2019), it is likely that the Mediterranean invader populations hosts local, thermally adapted symbionts (Schmidt et al., 2016). Unlike in foraminifera, the photosymbiotic relationship between corals and their zooxanthellae is more specific. Previous studies reported massive bleaching of symbiont-bearing corals after natural exposure to extremely cold temperatures (Hoegh-Guldberg et al., 2005; Lirman et al., 2011), and there are experimental observations of symbiont stress under cold exposure, expressed by decreased photosynthetic efficiency, loss of symbiotic algae and changes in concentrations of their photosynthetic pigments (Nielsen et al., 2020; Saxby, Dennison, & Hoegh-Guldberg, 2003). The degree of cold sensitivity controls poleward range expansion of corals both in tropical and temperate regions. Some species expand far faster than others (Yamano, Sugihara, & Nomura, 2011), and there is an evident gradient in species composition over the latitudinal range (Greenstein & Pandolfi, 2008). Moreover, at least for some species the range expansion is accompanied by reduced genetic diversity of the symbionts (Grupstra et al., 2017). Hence, the leading edge of corals might be limited by the specific host-symbiont relationship. The observed response (physiological and molecular) of the LBF diatom symbionts for coping with cold stress might suggest an advantage for range expansion compared to other symbiont-bearing tropical calcifiers such as corals, explaining their rapid invasion (Guastella et al., 2019) and proliferation (Weinmann, Rödder, Lötters, & Langer, 2013) in the new habitat.

Funding Information

This work was supported by ISF grant no. 941/17, and BMBF-MOST Cooperation in Marine Sciences grant numbers: 3-15275 and 3-15274. The authors thank the support provided to D.T. by the Mediterranean Sea Research Centre of Israel.

Supplementary Material

The following files are available in the digital form of this dissertation

Supplementary Figure 1. Output of GO_MWU for the 15°C versus 25°C comparison. Gene ontology (GO) terms with smaller fonts have a p-value < 0.01, while those with a larger, bold font have a p-value < 0.001. Red and blue text signifies GO terms that are upregulated or downregulated (respectively) at 15°C compared to at 25°C. The dendrogram on the left represents a clustering of terms based on their functional and expression similarities. Yellow arrows represent terms related to photosynthesis; these are the terms used to find enriched genes in Figure 4.

Supplementary Table S1. Details of all of the databases used to identify foraminifera and diatom sequences.

Supplementary Table S2. Mapping statistics of RNA-Seq data onto the transcriptome.

Supplementary Table S3. Summary of goseq analyses from the holobiont dataset. The terms represent biological processes with a false discovery rate adjusted p-value < 0.05.

Supplementary Table S4. Summary of goseq analyses from the symbiont dataset. All terms have a false discovery rate adjusted p-value < 0.05.

References

Alve, E., & Goldstein, S. T. (2003). Propagule transport as a key method of dispersal in benthic foraminifera (Protista). Limnology and Oceanography, 48(6), 2163–2170. doi: 10.4319/lo.2003.48.6.2163

Bowler, C., Allen, A. E., Badger, J. H., Grimwood, J., Jabbari, K., Kuo, A., … Grigoriev, I. V. (2008). The Phaeodactylum genome reveals the evolutionary history of diatom genomes. Nature, 456(7219), 239–244. doi: 10.1038/nature07410

Bryant, D. M., Johnson, K., DiTommaso, T., Tickle, T., Couger, M. B., Payzin-Dogru, D., … Whited, J. L. (2017). A Tissue-Mapped Axolotl De Novo Transcriptome Enables Identification of Limb Regeneration Factors. Cell Reports, 18(3), 762–776. doi: 10.1016/j.celrep.2016.12.063

Camacho, C., Coulouris, G., Avagyan, V., Ma, N., Papadopoulos, J., Bealer, K., & Madden, T. L. (2009). BLAST+: architecture and applications. BMC Bioinformatics, 10(1), 421. doi: 10.1186/1471-2105-10-421

Castillo, K. D., Ries, J. B., Bruno, J. F., & Westfield, I. T. (2014). The reef-building coral Siderastrea siderea exhibits parabolic responses to ocean acidification and warming. Proceedings of the Royal Society B: Biological Sciences, 281(1797), 20141856. doi: 10.1098/rspb.2014.1856

Cziesielski, M. J., Schmidt-Roach, S., & Aranda, M. (2019). The past, present, and future of coral heat stress studies. Ecology and Evolution, 9(17), 10055–10066. doi: 10.1002/ece3.5576

De Deckker, P. (2016). The Indo-Pacific Warm Pool: critical to world oceanography and world climate. Geoscience Letters, 3(1), 20. doi: 10.1186/s40562-016-0054-3

Doo, S. S., Mayfield, A. B., Byrne, M., Chen, H.-K., Nguyen, H. D., & Fan, T.-Y. (2012). Reduced expression of the rate-limiting carbon fixation enzyme RuBisCO in the benthic foraminifer Baculogypsina sphaerulata holobiont in response to heat shock. Journal of Experimental Marine Biology and Ecology, 430–431, 63–67. doi: 10.1016/j.jembe.2012.06.025

Dupuy, C., Rossignol, L., Geslin, E., & Pascal, P.-Y. (2010). Predation of mudflat meio-macrofaunal metazoans by a calcareous foraminifer, ammonia tepida (Cushman, 1926). The Journal of Foraminiferal Research, 40(4), 305–312. doi: 10.2113/gsjfr.40.4.305

Elith, J., & Leathwick, J. R. (2009). Species Distribution Models: Ecological Explanation and Prediction Across Space and Time. Annual Review of Ecology, Evolution, and Systematics, 40(1), 677–697. doi: 10.1146/annurev.ecolsys.110308.120159

Fabry, V. J. (2008). OCEAN SCIENCE: Marine Calcifiers in a High-CO2 Ocean. Science, 320(5879), 1020–1022. doi: 10.1126/science.1157130

Gertman, I., & Hecht, A. (2002). Tracking long-term hydrological change in the Mediterranean Sea. Monaco, 16, 75–78.

Glöckner, G., Hülsmann, N., Schleicher, M., Noegel, A. A., Eichinger, L., Gallinger, C., … Schliwa, M. (2014). The Genome of the Foraminiferan Reticulomyxa filosa. Current Biology, 24(1), 11–18. doi: 10.1016/j.cub.2013.11.027

Greenstein, B. J., & Pandolfi, J. M. (2008). Escaping the heat: range shifts of reef coral taxa in coastal Western Australia. Global Change Biology, 14(3), 513–528. doi: 10.1111/j.1365-2486.2007.01506.x

Grupstra, C. G. B., Coma, R., Ribes, M., Leydet, K. P., Parkinson, J. E., McDonald, K., … Coffroth, M. A. (2017). Evidence for coral range expansion accompanied by reduced diversity of Symbiodinium genotypes. Coral Reefs, 36(3), 981–985. doi: 10.1007/s00338-017-1589-2

Guastella, R., Marchini, A., Caruso, A., Cosentino, C., Evans, J., Weinmann, A. E., … Mancin, N. (2019). “Hidden invaders” conquer the Sicily Channel and knock on the door of the Western Mediterranean sea. Estuarine, Coastal and Shelf Science, 225, 106234. doi: 10.1016/j.ecss.2019.05.016

Habura, A., Hou, Y., Reilly, A. A., & Bowser, S. S. (2011). High-throughput sequencing of Astrammina rara: Sampling the giant genome of a giant foraminiferan protist. BMC Genomics, 12(1), 169. doi: 10.1186/1471-2164-12-169

Hallock, P., Lidz, B. H., Cockey-Burkhard, E. M., & Donnelly, K. B. (2003). Foraminifera as bioindicators in coral reef assessment and monitoring: the FORAM Index. Foraminifera in Reef Assessment and Monitoring. Environmental Monitoring and Assessment, 81(1–3), 221–238.

Henschel, R., Nista, P. M., Lieber, M., Haas, B. J., Wu, L.-S., & LeDuc, R. D. (2012). Trinity RNA-Seq assembler performance optimization. Proceedings of the 1st Conference of the Extreme Science and Engineering Discovery Environment on Bridging from the EXtreme to the Campus and beyond - XSEDE ’12, 1. New York, New York, USA: ACM Press. doi: 10.1145/2335755.2335842

Hoegh-Guldberg, O., Fine, M., Skirving, W., Johnstone, R., Dove, S., & Strong, A. (2005). Coral bleaching following wintry weather. Limnology and Oceanography, 50(1), 265–271. doi: 10.4319/lo.2005.50.1.0265

Hollaus, S. S., & Hottinger, L. (1997). Temperature dependence of endosymbiotic relationships? Evidence from the depth range of Mediterranean Amphistegina lessonii (Foraminiferida) truncated by the thermocline. Ecologae Geologicae Helvetiae, 90, 591–597.

Hughes, T. P., Kerry, J. T., Álvarez-Noriega, M., Álvarez-Romero, J. G., Anderson, K. D., Baird, A. H., … Wilson, S. K. (2017). Global warming and recurrent mass bleaching of corals. Nature, 543(7645), 373–377. doi: 10.1038/nature21707

Hyams-Kaphzan, O., L., P. G., & Almogi-Labin, A. (2014). Characteristics of benthic foraminifera inhabiting rocky reefs in northern Israeli Mediterranean shelf. Israel.

Kanehisa, M., & Goto, S. (2000). KEGG: Kyoto Encyclopedia of Genes and Genomes. Nucleic Acids Research, 28(1), 27–30. doi: 10.1093/nar/28.1.27

Kitazato, H. (1988). Ecology of benthic foraminifera in the tidal zone of a rocky shore. Revue de Paléobiologie, 2, 815–825.

Langer, M. R., & Hottinger, L. (2000). Biogeography of selected “larger” foraminifera. Micropaleontology, 46(SUPPLEMENT 1), 105–126.

Langmead, B., & Salzberg, S. L. (2012). Fast gapped-read alignment with Bowtie 2. Nature Methods, 9(4), 357–359. doi: 10.1038/nmeth.1923

Lesser, M. P. (1997). Oxidative stress causes coral bleaching during exposure to elevated temperatures. Coral Reefs, 16, 187–192.

Li, B., & Dewey, C. N. (2011). RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. BMC Bioinformatics, 12(1), 323. doi: 10.1186/1471-2105-12-323

Lirman, D., Schopmeyer, S., Manzello, D., Gramer, L. J., Precht, W. F., Muller-Karger, F., … Thanner, S. (2011). Severe 2010 Cold-Water Event Caused Unprecedented Mortality to Corals of the Florida Reef Tract and Reversed Previous Survivorship Patterns. PLoS ONE, 6(8), e23047. doi: 10.1371/journal.pone.0023047

Lommer, M., Specht, M., Roy, A.-S., Kraemer, L., Andreson, R., Gutowska, M. A., … LaRoche, J. (2012). Genome and low-iron response of an oceanic diatom adapted to chronic iron limitation. Genome Biology, 13(7), R66. doi: 10.1186/gb-2012-13-7-r66

Love, M. I., Huber, W., & Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biology, 15(12), 550. doi: 10.1186/s13059-014-0550-8

Murray, J. W. (2006). Ecology and Applications of Benthic Foraminifera. Cambridge: Cambridge University Press. doi: 10.1017/CBO9780511535529

Nielsen, J. J. V., Kenkel, C. D., Bourne, D. G., Despringhere, L., Mocellin, V. J. L., & Bay, L. K. (2020). Physiological effects of heat and cold exposure in the common reef coral Acropora millepora. Coral Reefs, 39(2), 259–269. doi: 10.1007/s00338-019-01881-x

Pinko, D., Abramovich, S., & Titelboim, D. (2020). Foraminiferal holobiont thermal tolerance under future warming – roommate problems or successful collaboration? Biogeosciences, 17(8), 2341–2348. doi: 10.5194/bg-17-2341-2020

Poloczanska, E. S., Burrows, M. T., Brown, C. J., García Molinos, J., Halpern, B. S., Hoegh-Guldberg, O., … Sydeman, W. J. (2016). Responses of Marine Organisms to Climate Change across Oceans. Frontiers in Marine Science, 3. doi: 10.3389/fmars.2016.00062

Prazeres, M., Morard, R., Roberts, T. E., Doo, S. S., Jompa, J., Schmidt, C., … Kucera, M. (2020). High dispersal capacity and biogeographic breaks shape the genetic diversity of a globally distributed reef-dwelling calcifier. Ecology and Evolution, ece3.6335. doi: 10.1002/ece3.6335

Prazeres, M., & Renema, W. (2019). Evolutionary significance of the microbial assemblages of large benthic Foraminifera. Biological Reviews, 94(3), 828–848. doi: 10.1111/brv.12482

Reiss, Z., & Hottinger, L. (1984). The Gulf of Aqaba: Ecological micropaleontology. In SPRINGER-VERLAG, NEW YORK, NY(USA). 1984. Berlin Heidelberg: Berlin Heidelberg: Springer-Verlag.

Ries, J. B., Cohen, A. L., & McCorkle, D. C. (2009). Marine calcifiers exhibit mixed responses to CO2-induced ocean acidification. Geology, 37(12), 1131–1134. doi: 10.1130/G30210A.1

Root, T. L., Price, J. T., Hall, K. R., Schneider, S. H., Rosenzweig, C., & Pounds, J. A. (2003). Fingerprints of global warming on wild animals and plants. Nature, 421(6918), 57–60. doi: 10.1038/nature01333

Rosenberg, E., & Ben-Haim, Y. (2002). Microbial diseases of corals and global warming. Environmental Microbiology, 4(6), 318–326. doi: 10.1046/j.1462-2920.2002.00302.x

Ross, B. J., & Hallock, P. (2016). Dormancy in the foraminifera: a review. The Journal of Foraminiferal Research, 46(4), 358–368. doi: 10.2113/gsjfr.46.4.358

Saxby, T., Dennison, W., & Hoegh-Guldberg, O. (2003). Photosynthetic responses of the coral Montipora digitata to cold temperature stress. Marine Ecology Progress Series, 248, 85–97. doi: 10.3354/meps248085

Schmidt, C., Morard, R., Prazeres, M., Barak, H., & Kucera, M. (2016). Retention of high thermal tolerance in the invasive foraminifera Amphistegina lobifera from the Eastern Mediterranean and the Gulf of Aqaba. Marine Biology, 163(11), 228. doi: 10.1007/s00227-016-2998-4

Schmidt, C., Morard, R., Romero, O., & Kucera, M. (2018). Diverse Internal Symbiont Community in the Endosymbiotic Foraminifera Pararotalia calcariformata: Implications for Symbiont Shuffling Under Thermal Stress. Frontiers in Microbiology, 9. doi: 10.3389/fmicb.2018.02018

Seppey, M., Manni, M., & Zdobnov, E. M. (2019). BUSCO: Assessing Genome Assembly and Annotation Completeness. doi: 10.1007/978-1-4939-9173-0_14

Smith, S. V., & Key, G. S. (1975). Carbon dioxide and metabolism in marine environments. Limnology and Oceanography, 20(3), 493–495. doi: 10.4319/lo.1975.20.3.0493

Stanley, G. D., & Lipps, J. H. (2011). Photosymbiosis: The Driving Force for Reef Success and Failure. The Paleontological Society Papers, 17, 33–59. doi: 10.1017/S1089332600002436

Stuart-Smith, R. D., Edgar, G. J., & Bates, A. E. (2017). Thermal limits to the geographic distributions of shallow-water marine species. Nature Ecology & Evolution, 1(12), 1846–1852. doi: 10.1038/s41559-017-0353-x

Stuhr, M., Blank-Landeshammer, B., Reymond, C. E., Kollipara, L., Sickmann, A., Kucera, M., & Westphal, H. (2018). Disentangling thermal stress responses in a reef-calcifier and its photosymbionts by shotgun proteomics. Scientific Reports, 8(1), 3524. doi: 10.1038/s41598-018-21875-z

Stuhr, M., Meyer, A., Reymond, C. E., Narayan, G. R., Rieder, V., Rahnenführer, J., … Hallock, P. (2018). Variable thermal stress tolerance of the reef-associated symbiont-bearing foraminifera

Amphistegina linked to differences in symbiont type. Coral Reefs, 37(3), 811–824. doi: 10.1007/s00338-018-1707-9

Supek, F., Bošnjak, M., Škunca, N., & Šmuc, T. (2011). REVIGO Summarizes and Visualizes Long Lists of Gene Ontology Terms. PLoS ONE, 6(7), e21800. doi: 10.1371/journal.pone.0021800

Titelboim, D., Almogi-Labin, A., Herut, B., Kucera, M., Asckenazi-Polivoda, S., & Abramovich, S. (2019). Thermal tolerance and range expansion of invasive foraminifera under climate changes. Scientific Reports, 9(1), 4198. doi: 10.1038/s41598-019-40944-5

Travis, J. L., Kenealy, J. F., & Allen, R. D. (1983). Studies on the motility of the foraminifera. II. The dynamic microtubular cytoskeleton of the reticulopodial network of Allogromia laticollaris. The Journal of Cell Biology, 97(6), 1668–1676. doi: 10.1083/jcb.97.6.1668

Vergés, A., Steinberg, P. D., Hay, M. E., Poore, A. G. B., Campbell, A. H., Ballesteros, E., … Wilson, S. K. (2014). The tropicalization of temperate marine ecosystems: climate-mediated changes in herbivory and community phase shifts. Proceedings of the Royal Society B: Biological Sciences, 281(1789), 20140846. doi: 10.1098/rspb.2014.0846

Weinmann, A. E., Rödder, D., Lötters, S., & Langer, M. R. (2013). Traveling through time: The past, present and future biogeographic range of the invasive foraminifera Amphistegina spp. in the Mediterranean Sea. Marine Micropaleontology, 105, 30–39. doi: 10.1016/j.marmicro.2013.10.002

Wright, R. M., Aglyamova, G. V, Meyer, E., & Matz, M. V. (2015). Gene expression associated with white syndromes in a reef building coral, Acropora hyacinthus. BMC Genomics, 16(1), 371. doi: 10.1186/s12864-015-1540-2

Yamano, H., Sugihara, K., & Nomura, K. (2011). Rapid poleward range expansion of tropical reef corals in response to rising sea surface temperatures. Geophysical Research Letters, 38(4), n/a-n/a. doi: 10.1029/2010GL046474

Young, M. D., Wakefield, M. J., Smyth, G. K., & Oshlack, A. (2010). Gene ontology analysis for RNA-seq: accounting for selection bias. Genome Biology, 11(2), R14. doi: 10.1186/gb-2010-11-2-r14

Zenetos, A., Gofas, S., Verlaque, M., Çinar, M. E., García Raso, J. E. G., Bianchi, C. N., … Streftaris, N. (2010). Alien species in the Mediterranean Sea by 2010. A contribution to the application of European Union's Marine Strategy Framework Directive (MSFD). Part I. Spatial distribution. Mediterranean Marine Science, 11(2), 381–493. doi: 10.12681/mms.87

General conclusions

The main goal of this thesis was to investigate the evolutionary mechanisms of the invasion success of a foraminifera invader in the Mediterranean Sea despite the different temperature regime in the new location. In this context, the aim was to understand if the adaptations necessary to survive in the Mediterranean were already present in the foraminifera host and/or its symbionts from the source population in the Red Sea (niche conservatism concept) or if the invasion success was due to rapid emergence of new genetic adaptations or transgenerational acclimatization (i.e., between generations) in the invaders (inducing ecological niche shifts). The resilience to new environmental conditions was investigated with a focus on the genetic adaptation of the foraminifera host, its microbiome flexibility, and the physiological performance of both the foraminifera and its symbionts. To this end, a set of complementary methodologies were applied including population genetics, metabarcoding analyses, and physiological experiments, and the results were presented in three different chapters formatted as research articles to treat each research question separately.

Research Question 1*: Is the invasion (a) facilitated by or (b) did it induce genetic adaptation of the foraminifera hots or (c) did the source population sustain key pre-adaptations?*

The findings in Chapter 1 revealed that preadaptation, high dispersion capacity, and continuous re-seeding of the Mediterranean from the Red Sea all work together to enhance the invasion of the symbiont-bearing foraminifera *Amphistegina lobifera* in the Mediterranean Sea. The observed lack of genetic separation between the invasive Mediterranean populations and the source population in the Red Sea demonstrates that the pre-adaptation to invasion was widespread in the source population. This result was obtained by sequencing the SSU rRNA and ITS gene (for the first time examined in *A. lobifera* in this manuscript) of the different populations which showed a reduced intragenomic variability and higher genetic variation across individuals in the invading population. This indicates that the invasion process seems to affect the reproductive strategy of the invading populations with an increase in asexual reproduction in the Mediterranean Sea that would result in greater gene conversion and thereby lower average heterozygosity. Therefore, it appears that the invasion is linked to a long-term abandonment of sexual reproduction. This chapter offers a perspective on the trade-off for new colonizers because the rapid shift towards asexuality during invasion may represent a long-term loss of adaptive potential in the new habitat.

Research Question 2*: Is the local adaptation enhanced by beneficial microbiome associations (microalgal and bacterial) that the foraminifera acquired during invasion, or did they carry the same associations as the source population?*

In Chapter 2, we investigated the eukaryotic and bacterial microbiome of *A. lobifera* along the invasion gradient and their similarity to communities in the surrounding environment. The different host populations showed associations with different bacterial microbiomes and diatom sequence variants pointing to a flexible symbiosis during invasion. To investigate the mechanism behind the flexible microbiomes, the research questions was further fragmented into two sub questions: (*a) Do the foraminifera acquire new symbionts (microalgae and bacteria) from the surrounding environment in the invaded habitat (microbiome switching)? (b) Do the foraminifera shift the proportions in the internal pool of symbionts (microbiome shuffling)?*

The comparison between the foraminifera and the surrounding environment revealed that the symbiotic diatom sequence variants were either rare or not at all detected in the surrounding environment. Therefore, we conclude that the foraminifera does not seem to acquire its symbionts from the immediate surrounding environment. However, it is not possible to infer if the foraminifera may have acquired symbionts from different locations over the course of the migration or if symbionts perhaps were taken up during a different season. Finally, the results also point to the possibility that new strains of diatoms were evolving within the host across the invasive range, which could be passed vertically by asexual reproduction (see Chapter 1). This could explain the observed higher richness of symbiotic diatoms than free-living environmental diatoms. Furthermore, these findings could be an indication that the foraminifera can actively shift the proportion of the internal pool of symbionts and select its diatoms to preserve the most beneficial strains.

Research question 3*: Did the sustained exposure to colder winters in the invasive locations induce adaptations in the foraminiferal host and/or its symbionts, or is there an inherited pre-adaptation from the source population?*

The results in Chapter 3 were based on physiological experiments to test the tolerance of the foraminifera holobiont (host and its microalgal symbionts) to colder temperatures, as currently experienced in the Mediterranean Sea. By measuring photophysiology of symbionts (using PAM-pulse amplitude modulated fluorometry) and growth rates/ survivorship, it was documented that the foraminifera host and its symbionts respond in an unsynchronized manner. While the survival and performance of the host did not significantly differ between the invading and source populations (all populations showed low tolerance to cold temperatures < 16°C), the symbionts showed enhanced tolerance to cold temperatures in the invaders (~13°C). In particular, the population at the invasion front supports a photosymbiosis that is significantly tolerant to even colder temperatures (~10°C). Thus, the foraminifera holobiont in the invasion front is better physiologically adapted to colder winters. In summary, this chapter concludes that the invasion success seems to be rather a property of the (shifted) symbionts than of the host, which preserves a low cold-temperature tolerance that appears to be just sufficient to survive the invasion.

Outlook

The findings of this thesis contribute to increase our knowledge of the mechanisms regulating marine invasions in the Mediterranean Sea. The successful invasive foraminifera host *Amphistegina lobifera* is known to tolerate the warmer summer temperatures in the new location, even when never experiencing before in their native habitat in the Red Sea (Schmidt et al., 2016). This thesis reveals that such tolerance of the host does not occur on the colder end of the Mediterranean thermal range (*see result*: Chapter 3). When exposed to cold stress at ~ 13°C, which is ~9°C below the average winter temperature in their native location, the foraminifera host reduces its growth, ceases its pseudopodia movement, and seems to enter a dormancy stage. In parallel, the symbionts maintain their photosynthetic activity (*see result*: Chapter 3, Figures 2 and 4). Thus, the symbionts are likely mainly responsible for the tolerance to the cold winters experienced in the Mediterranean (Table 1). This result was confirmed in the Appendix Chapter (Titelboim et al., 2021), whose findings imply that under cold stress, cellular resources are allocated to the maintenance of photosynthesis. Interestingly, we discovered that the population at the invasive front hosts a substantially more cold-resistant photosymbiosis (~10°C, Table 1). This can be explained by the flexibility of the foraminifera

to host different diatom symbionts (Prazeres et al., 2021; *see result*: Chapter 2). Therefore, the consolidated findings here highlight that even without genetic adaptations of the host, they can expand their ecological niche by the formation of novel biotic interactions and acclimatization to new habitats.

Table 1. Summary of thermal adaptation of the invasive *Amphistegina lobifera* and its symbionts to the high seasonality in the Mediterranean Sea. Based on the physiological responses of the host (growth[1,3], calcification[2]) and the diatom symbionts (photosynthetic fluorescence activity[1,3])

Invasive *Amphistegina lobifera*	**Warm adaptation (30 – 32 °C)[1,2]**	**Cold adaptation (~15 °C)[2,3]**	**Colder stress (~10 °C)[3]**
Foraminifera host	✓ (All populations)	⊘ (All populations)	⊘ (All populations)
Diatom symbionts	✓ (All populations)	✓ (All populations)	✓ (Only Sicilian population)

[1](Schmidt et al., 2016); [2](Titelboim et al., 2019); [3]This thesis

The novel findings described above help to improve predictions on the future spread of this and other symbiont-bearing species. There are several research strategies to describe how biological invasions work. It is possible to (a) focus on the characteristics of the invasive species and (b) those of the ecosystems they have invaded, (c) how these two factors relate to one another, or (d) to differentiate the invasion process through time (Heger and Trepl, 2003). This thesis followed most of these strategies. The investigation of the genetic adaptation of the host and their flexible microbiome focused on the characteristics of the invading species. The investigation of the environmental DNA in the seawater and sediment samples focused on the characteristics of the invaded ecosystem. And the experiments to test the physiological tolerance to cold stress of the host and its symbionts focused on the relationship between the invading species and the invaded ecosystem. Each of these strategies may help to further our understanding of specific aspects of biological invasions (Heger and Trepl, 2003). For instance, the overall warmer temperatures due to global change might facilitate the entry of warm-adapted invasive species into new locations (Hickling et al., 2006; Schmidt et al., 2016;

Walther et al., 2002). However, despite their warm adaptation, the invasive species might not be physiologically adapted to higher seasonality, as it has never experienced it before. Our findings reveal that if the host had no means to adjust or switch its symbionts it would likely not be able to survive the severe winter temperatures. Therefore, the ability to switch/shuffle symbionts to better-adapted strains is a great advantage for enduring stressful environmental conditions (Grégoire et al., 2017; Jones and Berkelmans, 2010) and must be taken into consideration to improve the accuracy of predictions of future marine invasions in response to climate change (Hiddink et al., 2012).

The findings of this dissertation raise some further hypotheses. For instance, (i) are there new strains of diatoms evolving inside the foraminifera host?; (ii) is the foraminifera host able to actively control its symbiont pool when exposed to thermal stress by expelling less-adapted symbionts and increasing the proportion of the better-adapted ones?; (iii) (how) is the foraminifera host able to recover from the likely dormancy stage after a prolonged exposure to cold temperatures?; (iv) if so, considering that the foraminifera becomes a particle that is easily carried by the currents when it is in this dormancy stage, would that explain the particular invasion success of this species and its continued north- and westwards expansion in the Mediterranean Sea?; and finally (v) would the flexible symbiosis offer an indirect advantage to the host without the need of a (rapid) genetic adaptation of the host?

The speed at which this species colonizes new habitats and basically replaces the native benthic foraminifera fauna is remarkable (Weinmann et al., 2013; Guastella et al., 2019; Chapter 1 of this dissertation). But the central question is what would be the effective impact of such establishment on ecosystem functioning. Would the invasion impact biodiversity (Bax et al., 2003), or would the species enter a “vacant” ecological niche (Herbold and Moyle, 1986)? Negative ecological impacts of invasive species have been widely studied and may include competition with and predation on native species, hybridization with native species, modifications to ecological processes, a loss of biodiversity, and an increase in pests and diseases (Bax et al., 2003; Crooks, 1998; Grosholz, 2002; Katsanevakis et al., 2014; Mack et al., 2000). However, the positive impacts of invasive species are probably underestimated, as there is often a perception bias against invasive species (Katsanevakis et al., 2014). For instance, invasive species can also induce facilitation of native species (i.e., interaction between two species that results in an increase in the density or biomass of at least one of the species; Rodriguez, 2006). These mechanisms include direct (creation of novel habitat, replacement of

habitat, nutrient enrichment, food source diversification, novel hosts, pollination) and indirect (competitive release, predatory release) interactions (Rodriguez, 2006; and references therein). This highlights the importance of continuing investigations into the invasion processes, their mechanisms, and their direct and indirect consequences on ecosystem functioning.

Finally, useful extensions to the present study would be to assess the effects of invasion on the contribution to carbonate production. LBFs are significant carbonate producers in contemporary tropical ecosystems due to their large calcareous tests (ranging in size from one mm to a few centimeters). The LBFs alone produce over 5% of the annual present-day calcium carbonate in the world's reefs and shelf regions (0-200 m) and around 2.5% of the $CaCO_3$ of all oceans, with an estimated yearly output of at least 130 million tons of $CaCO_3$ (Hallock, 1981; Langer, 2008). Moreover, symbiont-bearing benthic foraminifera are often the primary producers of calcium carbonate grains that are deposited at beaches in the tropical Central and West Pacific, as well as in the East Indian Ocean (Hohenegger, 2006; Narayan et al., 2022). Empty tests released during reproduction or at death are entrained at the reef crest by waves and transported by currents (Hallock., 2002). Therefore, the presence of this prolific-calcifier in an area where it did not previously occur expands the range for calcification and accumulation in the sediment, likely resulting in a substantial rise in carbonate output in the Mediterranean Sea. Addressing this question would be an additional next step to highlight the important contribution of this successfully invading protist in the Mediterranean Sea.

References

Bax, N., Williamson, A., Aguero, M., Gonzalez, E., Geeves, W., 2003. Marine invasive alien species: A threat to global biodiversity. Mar Policy 27, 313–323. https://doi.org/10.1016/S0308-597X(03)00041-1

Crooks, J.A., 1998. Habitat alteration and community-level effects of an exotic mussel, Musculista senhousia. Mar Ecol Prog Ser 162, 137–152.

Grégoire, V., Schmacka, F., Coffroth, M.A., Karsten, U., 2017. Photophysiological and thermal tolerance of various genotypes of the coral endosymbiont Symbiodinium sp. (Dinophyceae). J Appl Phycol 29, 1893–1905. https://doi.org/10.1007/s10811-017-1127-1

Grosholz, E., 2002. Ecological and evolutionary consequences of coastal invasions. Trends Ecol Evol 17, 22–27. https://doi.org/10.1016/S0169-5347(01)02358-8

Guastella, R., Marchini, A., Caruso, A., Cosentino, C., Evans, J., Weinmann, A.E., Langer, M.R., Mancin, N., 2019. "Hidden invaders" conquer the Sicily Channel and knock on the door of the Western Mediterranean sea. Estuar Coast Shelf Sci 225, 106234. https://doi.org/10.1016/j.ecss.2019.05.016

Hallock., P., 2002. Larger foraminifera as contributors to carbonate beach sands., in: Robbins, L.L., Magoon, O.T., Ewing, L. (Eds.), Carbonate Beaches 2000. Conference Proceedings. ASCE American Society of Civil Engineers, Reston, pp. 97–98.

Hallock, P., 1981. Production of Carbonate Sediments by Selected Large Benthic Foraminifera on Two Pacific Coral Reefs. Journal of Sedimentary Research 51. https://doi.org/10.1306/212F7CB1-2B24-11D7-8648000102C1865D

Heger, T., Trepl, L., 2003. Predicting biological invasions. Biol Invasions 5, 313–321. https://doi.org/10.1023/b:binv.0000005568.44154.12

Hickling, R., Roy, D.B., Hill, J.K., Fox, R., Thomas, C.D., 2006. The distributions of a wide range of taxonomic groups are expanding polewards. Glob Chang Biol 12, 450–455. https://doi.org/10.1111/j.1365-2486.2006.01116.x

Hiddink, J.G., ben Rais Lasram, F., Cantrill, J., Davies, A.J., 2012. Keeping pace with climate change: What can we learn from the spread of Lessepsian migrants? Glob Chang Biol 18, 2161–2172. https://doi.org/10.1111/j.1365-2486.2012.02698.x

Hohenegger, J., 2006. The importance of symbiont-bearing benthic foraminifera for West Pacific carbonate beach environments. Mar Micropaleontol 61, 4–39. https://doi.org/10.1016/j.marmicro.2006.05.007

Jones, A., Berkelmans, R., 2010. Potential Costs of Acclimatization to a Warmer Climate: Growth of a Reef Coral with Heat Tolerant vs. Sensitive Symbiont Types. PLoS One 5, e10437-.

Katsanevakis, S., Wallentinus, I., Zenetos, A., Leppäkoski, E., Çinar, M.E., Oztürk, B., Grabowski, M., Golani, D., Cardoso, A.C., 2014. Impacts of invasive alien marine species on ecosystem services and biodiversity: A pan-European review. Aquat Invasions 9, 391–423. https://doi.org/10.3391/ai.2014.9.4.01

Langer, M.R., 2008. Assessing the contribution of foraminiferan protists to global ocean carbonate production, in: Journal of Eukaryotic Microbiology. pp. 163–169. https://doi.org/10.1111/j.1550-7408.2008.00321.x

Mack, R.N., Simberloff, D., Mark Lonsdale, W., Evans, H., Clout, M., Bazzaz, F.A., 2000. Biotic Invasions: Causes, Epidemiology, Global Consequences, and Control. Ecological Applications 10, 689–710. https://doi.org/https://doi.org/10.1890/1051-0761(2000)010[0689:BICEGC]2.0.CO;2

Munday, P.L., Warner, R.R., Monro, K., Pandolfi, J.M., Marshall, D.J., 2013. Predicting evolutionary responses to climate change in the sea. Ecol Lett 16, 1488–1500. https://doi.org/https://doi.org/10.1111/ele.12185

Narayan, G.R., Reymond, C.E., Stuhr, M., Doo, S., Schmidt, C., Mann, T., Westphal, H., 2022. Response of large benthic foraminifera to climate and local changes: Implications for future carbonate production. Sedimentology 69, 121–161. https://doi.org/10.1111/sed.12858

Prazeres, M., Roberts, T.E., Ramadhani, S.F., Doo, S.S., Schmidt, C., Stuhr, M., Renema, W., 2021. Diversity and flexibility of algal symbiont community in globally distributed larger benthic foraminifera of the genus Amphistegina. BMC Microbiol 21, 1–16. https://doi.org/10.1186/s12866-021-02299-8

Rodriguez, L.F., 2006. Can invasive species facilitate native species? Evidence of how, when, and why these impacts occur. Biol Invasions 8, 927–939. https://doi.org/10.1007/s10530-005-5103-3

Schmidt, C., Morard, R., Prazeres, M., Barak, H., Kucera, M., 2016. Retention of high thermal tolerance in the invasive foraminifera Amphistegina lobifera from the Eastern Mediterranean and the Gulf of Aqaba. Mar Biol 163. https://doi.org/10.1007/s00227-016-2998-4

Titelboim, D., Almogi-Labin, A., Herut, B., Kucera, M., Asckenazi-Polivoda, S., Abramovich, S., 2019. Thermal tolerance and range expansion of invasive foraminifera under climate changes. Sci Rep 9, 1–5. https://doi.org/10.1038/s41598-019-40944-5

Titelboim, D., Thangarjan, S., Raposo, D., Morard, R., Kucera, M., Ashckenazi-Polivoda, S., Almogi-Labin, A., Herut, B., Manda, S., Abramovich, S., Gold, D.A., Abdu, U., 2021. The transcriptomic signature of cold and heat stress in benthic foraminifera—Implications for range expansions of marine calcifiers. Funct Ecol 35, 2679–2690. https://doi.org/10.1111/1365-2435.13929

Walther, G.-R., Post2, E., Convey3, P., Menzel, A., Parmesank, C., Beebee, T.J.C., Fromentin#, J.-M., Hoegh-Guldberg, O., Bairlein, F., 2002. Ecological responses to recent climate change.

Weinmann, A.E., Rödder, D., Lötters, S., Langer, M.R., 2013. Traveling through time: The past, present and future biogeographic range of the invasive foraminifera Amphistegina spp. in the Mediterranean Sea. Mar Micropaleontol 105, 30–39. https://doi.org/10.1016/j.marmicro.2013.10.002