UNIVERSITAT DE BARCELONA

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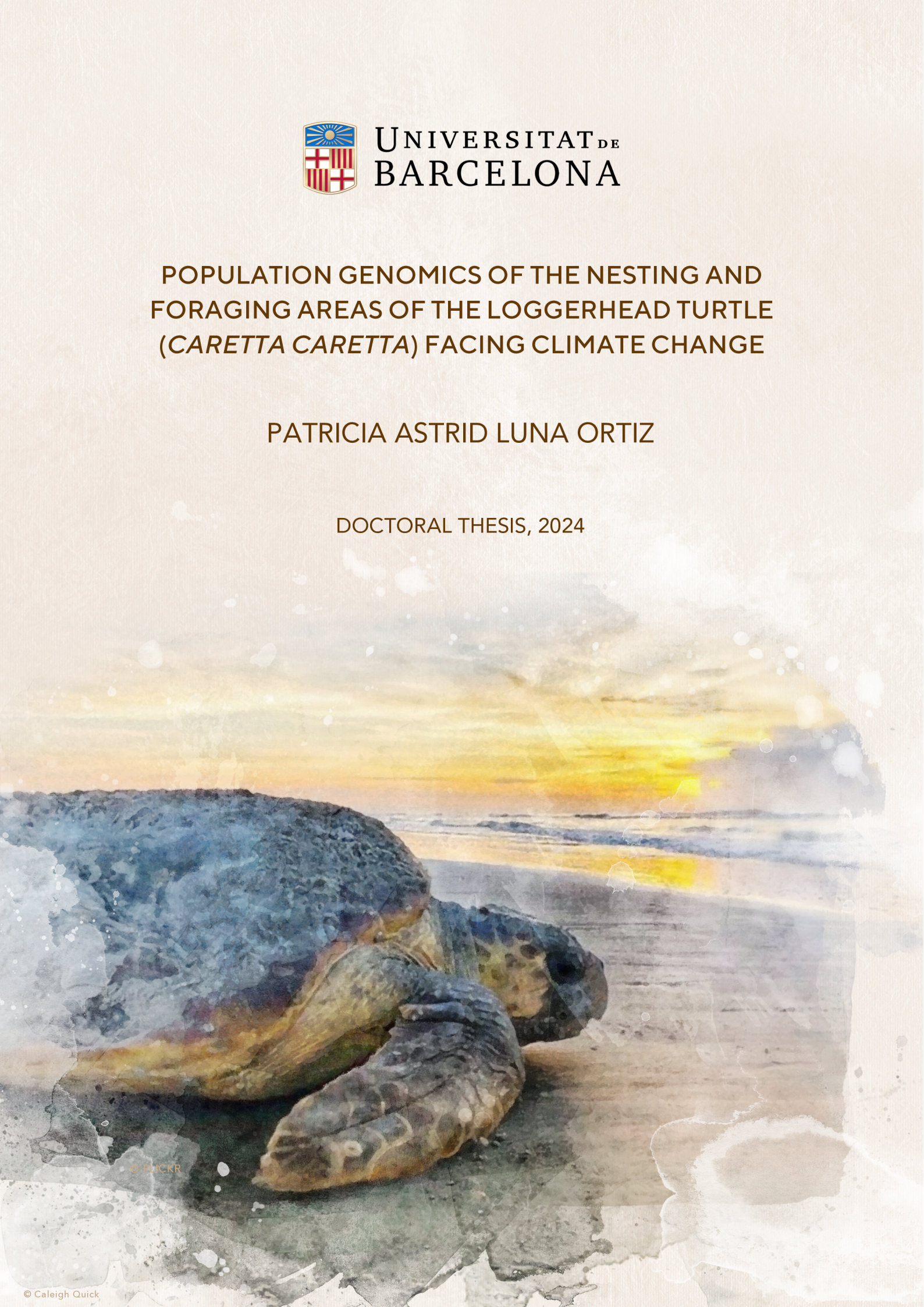
PATRICIA ASTRID LUNA ORTIZ

DOCTORAL THESIS, 2024



Faculty of Biology
Department of Genetics, Microbiology and Statistics
Doctoral program in Genetics

POPULATION GENOMICS OF THE NESTING AND FORAGING AREAS OF THE LOGGERHEAD TURTLE (*CARETTA CARETTA*) FACING CLIMATE CHANGE

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To qualify for the

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"*A goal without a plan is just a wish.*"

Antoine De Saint-Exupe

To Marco and Maximo Oliva, with all the love in my heart...

”*When the mystery is too overpowering, one dare not to disobey*”

—Antoine De Saint-Exupe

ABSTRACT

Genomic techniques are increasingly being utilized in conservation biology, providing critical insights into the behavior, reproductive success, and various threats faced by vulnerable species. The loggerhead sea turtle (*Caretta caretta*) is particularly sensitive to climate change and presents a complex life cycle characterized by long migrations from nesting populations to foraging grounds. The threats and characteristics of the species highlight the importance of understanding its population dynamics for informed management decisions and effective conservation strategies.

In this study, we focused on loggerhead turtles at different life stages. We first established a robust genomic baseline using the 2bRAD technique, analyzing 278 individuals from three regional management units (RMUs): the Mediterranean RMU (MED), represented by individuals from 11 nesting sites; the North West Atlantic RMU (NWA), represented by individuals from two nesting sites; and the North East Atlantic RMU (NEA), represented by individuals from one nesting site. We revealed significant genetic differentiation among the three RMUs and subsequently within the Mediterranean RMU identified three genetically differentiated SubRMUs: Greece (GRE), Levantine (LEV), and Sirte (SIR).

Additionally, we tested our baseline assessing the natal origin of juvenile loggerhead turtles in foraging areas employing a hierarchical approach, moving from a global perspective that included all three RMUs to a regional level that considered the three SubRMUs within the Mediterranean. We genotyped 103 individuals from four Mediterranean foraging areas: the Catalan region (CAT), Lampedusa (LAM), Eastern Aegean Sea (EAS), and Western Aegean Sea (WAS). Only 3 individuals were assigned to

NWA and 1 to NEA, thus indicating that these foraging areas aere primarly used by Mediterranean individuals. Among the 99 individuals assigned to the Mediterranean RMU, the individuals assigned to the Lebantine SubRMU were predominant, with some exhibiting mixed assignments, indicating some admixture among subregions.

Finally, to assess the rapid increase in the number of nesting events observed in Spain, we sampled 45 hatchlings from eight nests laid between 2016 and 2019. We found that nests were laid by different females, except for two nests attributed to the same female but within the same season. This suggests that the nesting activity is due to a surge of colonizing individuals rather than a return of females born in the region. We hypothesize that the rising number of colonizers results from successful conservation efforts, a feminization of the originating populations, and earlier sexual maturation among individuals. Using our baseline, we identified in the emerging nesting area, one hybrid nest between an Atlantic female and a Mediterranean male assigned to the Sirte SubRMU, as well as several nests resulting from the genetic admixture among different Mediterranean SubRMUs. Thus, in response to climate change, the expansion into new nesting sites may be promoting genetic mixing between previously isolated populations, with potential implications for the species' conservation. Our results not only clarify the current status of this colonization, but also highlight the need for ongoing efforts to monitor returning individuals to confirm the establishment of a stable resident population.

In conclusion, the results obtained in this study provide critical information for understanding the ecological and evolutionary processes affecting loggerhead sea turtles. Our findings will contribute to enhancing conservation strategies in long lived species with high and complex dispersal behavior by identifying populations impacted by threats beyond nesting areas and facilitating the study of specific origins in mixed foraging habitats.

RESUMEN

La tortuga boba (*Caretta caretta*) es la tortuga marina más abundante en el Mar Mediterráneo y presenta un ciclo de vida complejo entre zonas de anidación y de alimentación. Evaluar su estructuración poblacional, identificar el origen natal de los juveniles en zonas de alimentación y analizar la nidificación emergente como resultado del cambio climático permitirá diseñar estrategias de gestión adecuadas. El reciente desarrollo de herramientas genómicas para a especie, como el 2bRAD, permite abordar estas cuestiones a pesar de la complejidad que presenta la especie.

En esta tesis, se utilizó esta técnica genómica para construir un baseline genómico de las tres Unidades de Manejo Regional (RMU) que pueden contribuir con individuos presentes en el Mediterráneo: Atlántico-Noroeste (NWA), Atlántico-Noreste (NEA) y Mediterráneo (MED) Se identificó una diferenciación genética significativa entre las 3 RMUs y dentro del Mediterráneo se detectaron tres SubRMUs: Grecia, Levante y Sirte. El baseline se utilizó para asignar juveniles de las zonas de alimentación tanto a nivel de RMU como SubRMU con alta precisión (>0.7), y se detectaron individuos de origen mixto entre SubRMUs.

Asimismo, se evaluó el aumento de anidaciones en España analizando 45 crías de 8 nidos (2016-2019) con la misma metodología genómica. Se identificaron diferentes hembras nidificantes excepto por dos nidos puestos por la misma hembra durante la misma época de nidificación. Utilizando nuestro baseline, identificamos que uno de los nidos era mixto entre una hembra atlántica y un macho de origen mediterráneo, así como múltiples nidos resultantes de la reproducción entre individuos de diferentes SubRMUs mediterráneas. Los resultados sugieren que la anidación se debe a nuevos

individuos colonizadores y no a tortugas remigrantes. Hipotetizamos que esta colonización se relaciona con esfuerzos exitosos de conservación, feminización de la población y una maduración sexual temprana, y dado el alto porcentaje de crías hembras sugerimos el potencial establecimiento de una población residente en la región. El uso de herramientas genómicas como 2bRAD mejora la comprensión de los procesos evolutivos y ecológicos que afectan a especies vulnerables. Los resultados de esta tesis son clave para decisiones informadas en conservación de esta especie y enfrentar sus desafíos frente al cambio climático y la colonización de nuevas áreas.

CONTENTS

01 INTRODUCTION

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1. INTRODUCTION

1.1 Climate change in the world

Climate variability includes not only spatial differences but also temporal fluctuations. Historical data reveals that climate has experienced transitions between warmer and cooler periods, as well as between wetter and drier conditions. These transitions are supported by indirect evidence such as seafloor spreading, pollen records, glacial ice cores, and tree ring analyses (Hamza et al., 2020).

Since the last century, the global surface temperature has risen by approximately 1°C. At the same time, there has been a notable reduction in ice and snow cover in the Northern Hemisphere, a decrease in the Greenland ice sheet, rising sea levels, and shifts in the distribution of temperature-sensitive species towards higher latitudes, altogether providing evidence of significant climatic changes (Ogunbode et al., 2020).

Climate change arises from both natural processes and anthropogenic activities. Natural drivers include variations in Earth's orbital parameters, which alter solar energy distribution, and plate tectonics, which affect Earth's energy balance by redistributing landmasses (Schurer et al., 2014). Human activities, such as deforestation, industrial processes, and aerosol emissions, have significantly increased greenhouse gas concentrations (Irving et al., 2019). The United Nations Framework Convention on Climate Change (UNFCCC) classifies climate change as predominantly human-induced, while variability refers to changes driven by natural factors (Solomon et al., 2007).

1.1.1 Climate change in the Oceans

Rapid global warming has strongly impacted weather patterns, climate systems, ecosystems, human societies, and economies. The warming climate is particularly evident in the oceans, where rising temperatures have been linked to the reduction in ice sheet, increase in global mean sea level, altered hydrological cycles, and modifying atmospheric and oceanic circulation (Hamza et al., 2020).

Associated with global warming, the increasing concentration of atmospheric carbon dioxide (CO_2) has a direct impact on ocean chemistry through CO_2 absorption by surface waters. This process reduces ocean pH, leading to ocean acidification, hindering the ability of marine organisms such as corals, oysters, and pteropods to form calcium carbonate shells and skeletons (Lemasson et al., 2017). Furthermore, acidification has been shown to reduce the fitness of species such as coccolithophores, crabs, sea urchins, and early-stage fish (Tasoff & Johnson, 2018). Over the past decade, ocean acidification, combined with other stressors suche as eutrophication, warming, and hypoxia, has been studied to affect marine species, ecosystems, and biogeochemical cycles (Baumann, 2019)

Oxygen is critical for oceanic life, defining habitats for marine organisms. Oxygen can only be introduced to the upper ocean layers through photosynthesis or air-sea gas exchange. As water moves away from the surface, oxygen levels decrease due to consumption. Global warming exacerbates this by reducing oxygen solubility at the surface, lowering the initial levels of subducted oxygen. Additionally, warming affects biological activity and ocean stratification, all of which may further decrease oceanic oxygen levels (Stramma & Schmidtko, 2021).

The sea ice at the poles maintaining the global heat balance is also an important factor. The high reflectivity (albedo) of sea ice reflects solar energy and radiates long-wave

heat, helping to regulate global temperatures. However, the loss of ice diminishes the albedo, enabling greater heat transfer from the ocean to the atmosphere through thinner ice and expanding areas of open water, thereby weakening Earth's capacity to sustain its heat balance, in addition to significantly impact the ocean's salinity balance (Polyakov et al., 2020).

The oceans are undergoing processes significantly related to climate change and assessing the marine wildlife adaptation is of importance for preserving the biodiversity. To do this, it is important to study the effects of temperature increases on a smaller scale, which allows the results to be extrapolated to other regions and even to other organisms.

1.1.2 Climate change in the Mediterranean and Atlantic Sea

On a regional scale, the Mediterranean Sea, considered a 'hotspot', is regarded as one of the most important and at-risk areas globally concerning climate change (Cramer et al., 2018). This sea is surrounded by Africa, Europe and Asia, and is divided into two sub-divisions (Western and Eastern) by a sill that does not exceed 400 meters in depth, located between Sicily and the African continent (Boxer & Salah, 2024) (Fig. 1).

The Mediterranean Sea was one of the first seas which a warming trend in deep water temperatures in the Western Basin was directly attributed to global warming (Bethoux et al., 1990). Since the mid-1980s, the Mediterranean sea surface temperature (SST) has shown a pronounced and persistent warming trend, which is projected to continue as numerous studies have documented a steady increase in mean Mediterranean SST (Pastor et al., 2019).

For the climate of the Mediterranean region, natural modes of variability include the North Atlantic Oscillation (NAO, atmospheric pressure pattern that influences climate in

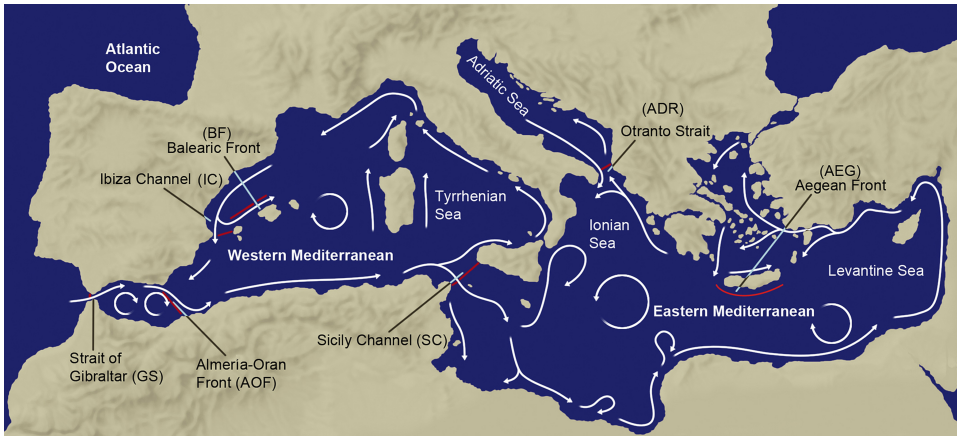

Figure 1: Map of the Mediterranean Sea with the name of the sub-basins, main currents (white lines) and oceanographic fronts (red lines). The name of the fronts and the acronym used (in black) is as follows: GS (Gibraltar Strait), AOF (Almeria-Oran Front), IC (Ibiza Channel), BF (Balearic Front), SC (Sicily Channel), ADR (Otranto Channel), AEG (Southern margin of the Aegean Sea). Figure from (Pascual et al., 2017)

the North Atlantic region) and the Atlantic Multidecadal Oscillation (AMO, long-term sea surface temperature variability across the North Atlantic), anomalies in these patterns lead to alterations such as that in a positive NAO phase, the western Mediterranean experiences warmer conditions, and cooler conditions are observed in the eastern Mediterranean; conversely, during negative NAO phases this pattern is reversed, affecting wind patterns, air temperature and precipitation, especially during winter and early spring (Trigo et al., 2002).

Recent research has explored long-term variations and trends in sea surface temperature (SST) in the Mediterranean Sea and the adjacent Northeast Atlantic from 1982 to 2018, using satellite data from the Copernicus Marine Environment Monitoring Service (CMEMS) and has identified a significantly increasing trend (mean, 0.041 ± 0.006 °C/yr) with notable differences within the region, particularly higher increases in the eastern Mediterranean, and more specifically in the Levant-Aegean basin compared to the western Mediterranean (Pisano et al., 2020) (Fig.2). A key finding was the divergence in temperature trends between the Mediterranean and the Northeast Atlantic after the 1990s, where the Mediterranean continued to exhibit warming, while the Northeast

Atlantic did not show a significant trend until 2015. Furthermore, it is highlighted that this warming trend affects both the overall SST and the seasonal cycle, characterized by increasing summer temperatures coupled with reducing winter temperatures (Pisano et al., 2020).

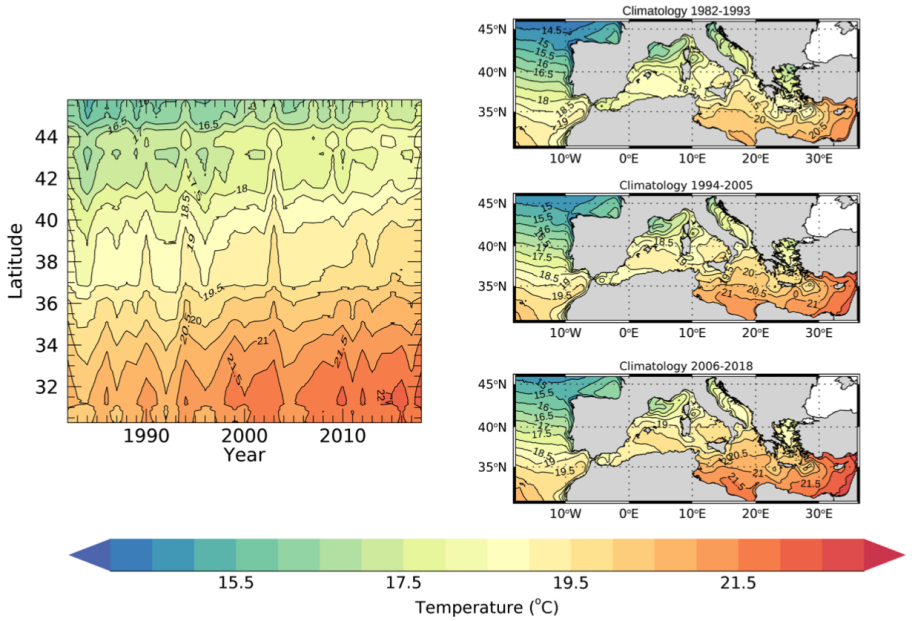

Figure 2: Long-term variations and trends in sea surface temperature (SST) in the Mediterranean Sea and North East Atlantic from 1982 to 2018 Left panel: Hovmöller diagram of the Sea Surface Temperature (SST) trends in the Mediterranean Sea (including part of the Northeastern Atlantic region) from 1982 to 2018. Right panel: (top) annual mean SST for the period 1982 to 1993; (middle) annual mean SST from 1994 to 2005; and (bottom) annual mean SST from 2006 to 2018. Figures from Pisano *et al.*, 2020 (Pisano et al., 2020).

1.1.3 Effects of climate change in sea species

Climate influences marine populations through a variety of ecological mechanisms, including reproduction, growth, migration patterns, recruitment, and phenology; major climate stressors affecting ocean ecosystems include warming, acidification, and deoxygenation (Gruber, 2011). The impacts of climate change may also extend to marine species life cycles, productivity, variability, seasonality, and distribution, potentially exacerbating challenges for already vulnerable communities (Pecl et al., 2017). While ocean warming is not uniform, a shift in species distribution linked to rising temperatures

has been already observed for some species (Pecl et al., 2017). Accelerated ocean warming could therefore influence the distribution, abundance, and life history traits of wildlife.

While research on the effects of climate change on biodiversity has focused primarily on terrestrial organisms, there is growing evidence of significant impacts on marine biodiversity, observed at both local and global scales (Worm & Lotze, 2021). Historically, long-term studies have centered on fish and plankton (Worm & Lotze, 2021), but recent research has expanded to include large marine mammals (Albouy et al., 2020), corals (Hughes et al., 2018), and sea turtles (Cardona et al., 2023), among others.

1.1.4 Climate change and sea turtles

Sea turtles represent an interesting model, as they have both marine and terrestrial stages and rely on productive ecosystems for foraging, as well as low-lying sandy beaches for nesting (Bolten et al., 2003). Given their global distribution across diverse marine habitats (Wallace et al., 2023), all seven species of sea turtles are likely to be impacted by climate change, with effects varying by geography, time, species, and population (Poloczanska et al., 2009).

In assessing the effects of climate change on sea turtles, key parameters that affect their survival and reproductive success have been described, to be consider when designing conservation strategies to mitigate the adverse effects of climate change on these vulnerable species and their ecosystems (Fig.3) (Patrício et al., 2021).

1. **Sex ratio**

   Climate change critically influences in sex ratios trough temperature dependent sex determination (TSD). As global temperatures rise, nesting sites experience higher incubation temperatures, leading to a disproportionate increase in female

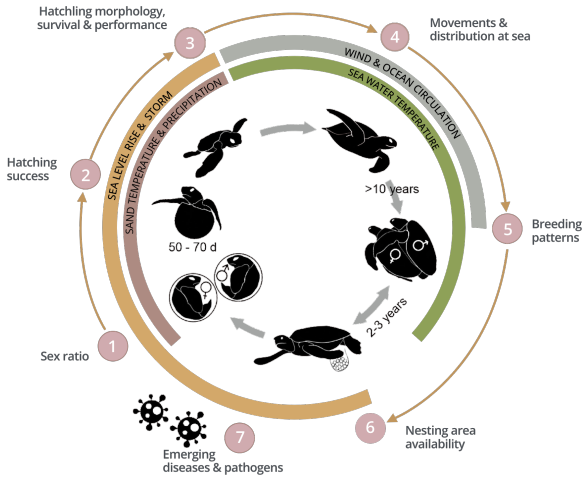

Figure 3: General life cycle of marine turtles and key parameters affected by climate change. Diagram of the major life stages of sea turtles, highlighting factors vulnerable to climate change. These threats together pose significant challenges to the long-term survival of sea turtles.

hatchlings. This skewed sex ratio threatens population stability by reducing the number of males available for reproduction, which can lead to long-term demographic imbalances. The trend toward feminization is particularly concerning for species already at risk, as it can decrease genetic diversity and resilience (Patrício et al., 2021).

2. **Hatching success**

   Hatching success depends on several factors, including incubation temperature, humidity, and environmental conditions. Success is typically above 65%, but can decrease significantly with small increases in temperature, especially above 30°C. Lethal thermal limits for embryos vary between species and are influenced by factors such as temperature, duration, humidity, and precipitation. Climate change exacerbates these problems by potentially increasing the frequency of extreme temperatures and weather events (Patrício et al., 2021).

3. **Hatching morphology, survival, and performance**

   Incubation temperature affects morphology, survival, and hatching performance.

Warmer incubation temperatures increase metabolic rates and biochemical reactions, influencing hatch size, locomotion, and scute abnormalities. Cooler temperatures may result in larger broods due to more efficient yolk conversion. Performance relationships with optimal performance incubation temperature occur at intermediate temperatures. Research on swimming performance and physiological differences suggests that warmer incubation temperatures may impair muscle function and swimming efficiency (Patrício et al., 2021).

4. **Movements and distribution at sea**

   Climate-induced changes in sea temperatures are altering the timing of key life events, such as nesting and migration, leading to mismatches between turtle behavior and optimal environmental conditions. These changes may impact hatching success, as altered nesting seasons may expose eggs to unfavorable temperatures or increased predation. Additionally, climate change is driving shifts in the geographic distribution of turtles, forcing them to adapt to new habitats or abandon the traditional areas (Patrício et al., 2021).

5. **Breeding patterns**

   Sea turtles exhibit significant variability in the timing and length of the nesting season, influenced by environmental conditions at both foraging and breeding sites. Cooler waters can improve food availability, leading to better breeding conditions, while short-term cues such as seawater temperature can trigger nesting. Climate change can impact migration, courtship, and nesting duration. Responses to climate change vary among species and regions, complicating predictions. It is important to consider multiple factors, including environmental cues, population demographics, and geographic differences, to understand sea turtle reproductive phenology and adapt conservation strategies accordingly (Patrício et al., 2021).

6. **Nesting area availability**

   Sea level rise (SLR) and increased storm activity threaten marine turtle nesting sites. SLR can reduce available nesting areas, increase density-dependent risks, and expose nests to saltwater inundation, affecting the hatching success (Patrício et al., 2021).

7. **Emerging diseases and pathogens**

   In recent decades, outbreaks of infectious diseases in marine species, including marine turtles, have increased, likely due to a combination of climate change and anthropogenic factors. Major concerns include fibropapillomatosis (FP), caused by a herpesvirus, and the emerging fungal disease, sea turtle egg fusariosis (STEF). Although FP is not currently a major threat, rising sea temperatures could exacerbate its severity. STEF, linked to *Fusarium* fungi, has been associated with increassed nest mortality, especially under stressors like tidal inundation. Climate change may further impact turtle health by affecting pathogen virulence and affecting vital habitats (Patrício et al., 2021).

In response to climate change, conservation strategies will need to encompass both intervention and mitigation. Intervention involves direct measures, such as relocating nests or modifying incubation conditions, while mitigation aims to reduce external stressors, allowing turtles to adapt naturally. However, interventions such as sex ratio manipulation or site protection must be carefully considered due to ecological risks and uncertainties. Effective management will require site-specific strategies, adaptive learning, and thorough assessments of long-term population impacts on the population. Understanding the genetic and ecological characteristics of each population is essential to accurately measure adaptive capacities and limitations (Patrício et al., 2021).

1.2 The loggerhead sea turtle (*Caretta caretta*)

The loggerhead sea turtle (Fig.4) is a medium-sized turtle from the family Cheloniidae that can reach a straight carapace length of 120 cm and a weight of 200 kg (Marco et al., 2009). It is distinguished by a large head with a robust beak and neck. The dorsal surface is brown with reddish or orange margins and the belly is whitish with pale yellow tones. It is characterized by having two pairs of prefrontal scales on the head. The dorsal carapace shows 5 vertebral scales, 5 costal scales, 11-13 marginal scales on each side and 2 supracaudal scales; the nuchal scale is in contact with the first two costal scales, although asymmetrical anomalies in the arrangement of the scales are common. The keratinized beak has smooth edges. The front flippers and the hind limbs, shaped like a rudder, have 2 claws. Adult males reach a size slightly larger than females and have a relatively long and robust tail that can exceed 30 cm in length, while in females it rarely protrudes from the carapace. These external secondary sexual characteristics do not appear until sexual maturity. Newborns have dark grey tones and usually have the vertebral scales overlapping forming 2 or 3 dorsal keels, as well as the serrated edge of the carapace, traits that are lost as the turtles approach sexual maturity. They acquire the reddish brown tones characteristic of the species in the young juvenile stage. In large individuals, the presence of a variety of epibiont organisms associated with the dorsal carapace is frequent, such as algae, tube worms, barnacles or other sessile crustaceans.

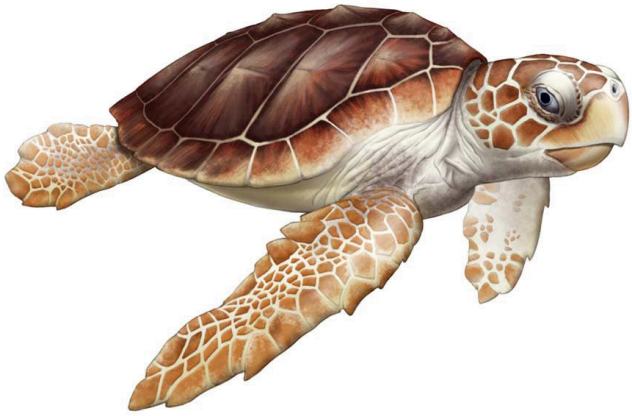

Figure 4: Loggerhead sea turtle (*Caretta caretta*). The loggerhead sea turtle is a species of marine reptile characterized by its broad head and strong jaw. It inhabits temperate and subtropical waters of the Atlantic, Pacific, Indian and Mediterranean oceans. This species is known for its longevity and its ability to migrate long distances between feeding areas and the beaches where it nests. It is currently considered a vulnerable species due to habitat loss, pollution and incidental capture in fishing nets (IUCN). Scientific illustration by: Juan Muñoz (https://juanm.net/).

1.2.1 Distribution of the loggerhead sea turtle

The loggerhead sea turtle has a broad global distribution using both oceanic/pelagic and neritic habitats, categorized into 9 confirmed Regional Management Units (RMUs) across multiple ocean basins, with a potential 10th RMU in the Northeast Indian Ocean, where data is still incomplete (Fig. 5). RMUs represent distinct population segments that are critical for conservation and management efforts (Wallace et al., 2023).

Each RMU have unique ecological conditions and threats, highlighting the need for region-specific conservation strategies. Furthermore, genetic research has revealed distinct genetic stocks within these RMUs, indicating significant population structure and connectivity within and across regions (Wallace et al., 2023). This genetic diversity emphasizes the complexity of loggerhead turtle populations and the importance of protecting both local and migratory populations. Conservation efforts must consider the varying threats faced by these RMUs, such as bycatch, habitat loss, and climate change, to ensure the long-term survival of loggerhead turtles across their global range.

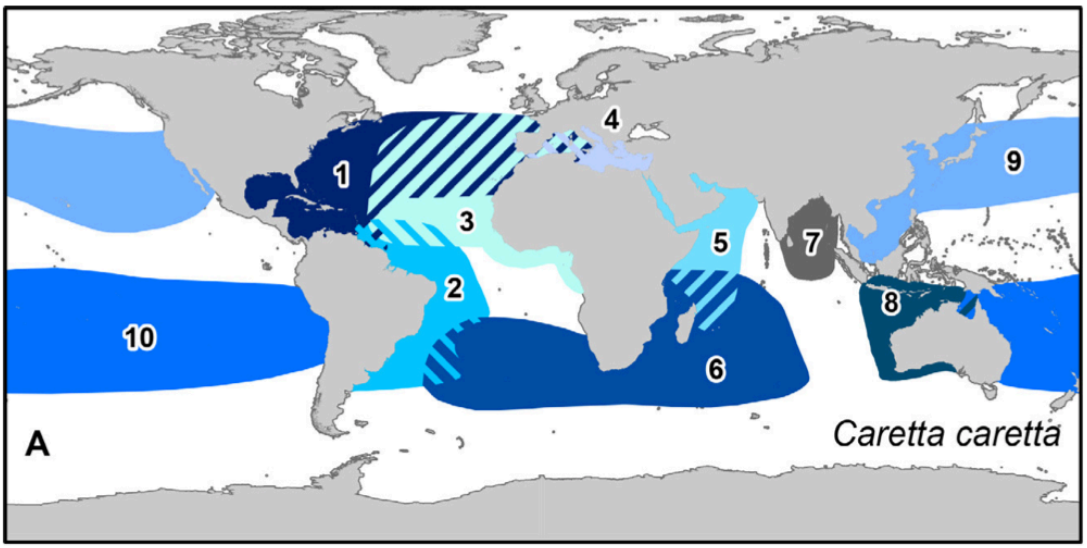

Figure 5: Regional Management Units (RMUs) for the Loggerhead sea turtle (*Caretta caretta*). Each of the Management Units is shown in a different shade of blue, with numbers indicating each of the regions. RMUs. **1**: Northwest Atlantic, **2**: Southwest Atlantic, **3**: Northeast Atlantic, **4**: Mediterranean, **5**: North- west Indian, **6**: Southwest Indian, **7**: Northeast Indian (assumed), **8**: Southeast Indian, **9**: North Pacific, **10**: South Pacific. RMUs were identified by georeferencing data on marine turtle bibliography, including nesting sites, population abundance and trends, population genetics, and satellite telemetry. Figure from (Wallace et al., 2023).

North West Atlantic

In the North West Atlantic, *C. caretta* predominantly nest along the U.S. coast, with primary nesting sites located in Florida (Shamblin et al., 2011). Additional nesting occurs in Mexico (González et al., 2020), Cuba (Moncada-Gavilán et al., 2014), and Central (Restrepo et al., 2022) and South America (Lima et al., 2012). After hatching, juveniles migrate offshore, often associating with Sargassum habitats where they find food and shelter as they drift through the North Atlantic gyre. Some juveniles are also known to enter the Mediterranean Sea during these early developmental stages. Over time, they establish long-term residency, reflecting the species' remarkable adaptability. As they grow, loggerheads transition to neritic zones, primarily occupying the continental shelf waters of the U.S. coast. Non-nesting adults are often found in estuaries that offer access to the open ocean and shallow waters, with significant populations observed in Florida Bay, the Bahamas, and Cuba (Ceriani & Meylan, 2017).

North East Atlantic

In the North East Atlantic, the Cape Verde population represents the most important nesting site for the species in the RMU, being one of the biggest global aggregations, together with Florida and Oman (Agyekumhene et al., 2017). Individuals display distinct migratory behaviors based on size, larger individuals tend to migrate to benthic foraging grounds off the coast of northwest Africa, while smaller individuals remain in the ocean, demonstrating varied foraging strategies (Oliwina et al., 2020). Juveniles from Cape Verde are commonly found in foraging areas such as the Canary Islands, Madeira, Andalusia, and even regions of Spain further into the Mediterranean Sea (Monzón-Argüello et al., 2009), genetic studies suggest that many may inhabit regions yet to be fully mapped (Godley et al., 2003).

Mediterranean

Loggerhead turtles represent the most abundant turtle species in the region. They are widespread throughout the Mediterranean Sea, with significant nesting historically concentrated in the eastern basin, especially in Cyprus, Greece, Turkey and Lybia (Casale, 2010). As a result of the species philopatry, a genetic structure is suggested with reduced gene flow among groups of rookeries including Israel (Carreras et al., 2006)

Essential neritic habitats for adult turtles are located on the continental shelves of Tunisia-Libya, the northern Adriatic Sea, Egypt, and Spain, with additional key foraging zones in Greece and Turkey. Juvenile forage in the south Adriatic, Ionian Sea, Sicily Strait, and Western Mediterranean. Migration patterns, tracked through tagging and satellite telemetry, reveal that females nesting in Greece migrate to the Gulf of Gabés and northern Adriatic, while those nesting in Cyprus travel towards Egypt and Libya, often repeating the same migration routes across seasons. Juvenile migrations span the Mediterranean and extend into the Eastern Atlantic, highlighting the species broad migratory range. In recent years the species has sporadically nested in the Western

Mediterranean with the number of nests raising in the last decade (Hochscheid et al., 2022)

1.2.2 The life cycle of the loggerhead turtle

Loggerhead turtles (*Caretta caretta*) have a complex life cycle with distinct stages, each characterized by specific habitats and behaviors that are essential to their survival. Each stage plays a critical role in the species development, with several environmental factors influencing their survival and growth (Fig.6) (Conant et al., 2009):

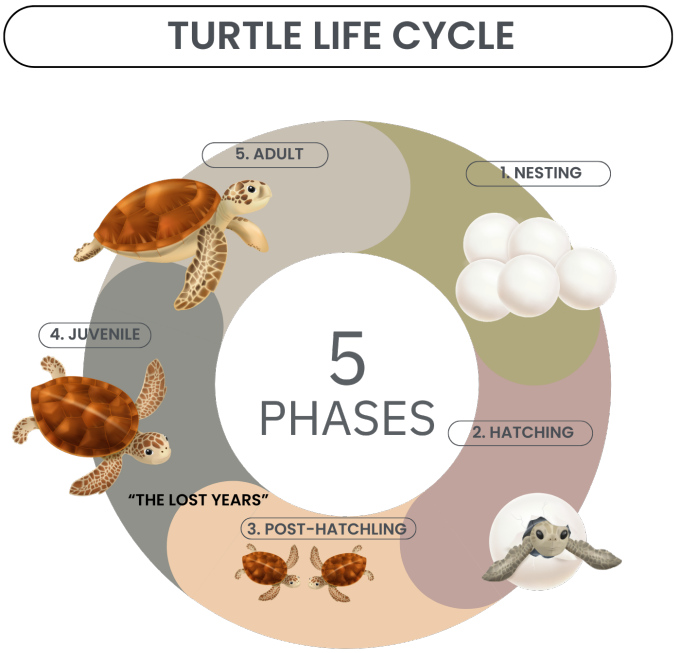

Figure 6: Life cycle of the loggerhead sea turtle (*Caretta caretta*). The life cycle of the species consist of key stages: **1. Nesting Stage**: Females lay between 100 and 130 eggs in nests on sandy beaches, with incubation periods ranging from 45 to 75 days. Nest temperature determines the sex of the hatchlings, with a critical temperature of 29°C for a balanced sex ratio, **2. Hatchling Stage**: Hatchlings emerge from the nest at night and head toward the ocean, swimming vigorously for several hours to move offshore and avoid predators, **3. Post-Hatchling Stage**: At this stage, hatchlings are pelagic, drifting in oceanic zones rich in Sargassum, where they find food and shelter, **4. Juvenile Stage**: Juveniles grow in oceanic environments for several years before migrating to neritic coastal zones, where they continue their maturation, **5. Adult Stage**: Adults inhabit coastal neritic zones, where they feed, migrate, and reproduce. Females often return to the same beaches to nest, exhibiting a behavior known as philopatry.

1. Nesting Stage

Loggerhead turtles typically nest on wide, sandy beaches, often backed by low dunes. Female lay clutches averaging 100 to 130 eggs per nest, with incubation periods varying from 45 to 75 days, depending on sand temperature. Incubation temperature is crucial as it determines the sex of the hatchlings, with warmer temperatures producing females and cooler temperatures producing males. The critical temperature for a balanced sex ratio is approximately 29°C. Nest humidity also affects hatching success, incubation duration, and hatchling size (Conant et al., 2009).

2. Hatchling Stage

After the incubation period, hatchlings emerge from the nest, usually at night when temperatures drop, triggering their emergence. They instinctively move towards the ocean, guided by natural light cues from the horizon. This journey from nest to sea is known as the s̈wimming frenzy,̈ a period of intense activity where hatchlings swim continuously for several hours to move offshore and avoid predators. Once in the open ocean, hatchlings transition to a low-energy float-and-wait strategy, feeding on small organisms associated with Sargassum and other floating debris (Conant et al., 2009).

3. Post-Hatchling Stage

At this stage, turtles are primarily pelagic, inhabiting oceanic areas where they drift with currents and accumulate in convergence zones rich in Sargassum. These areas provide both food and shelter. The post-hatchling stage lasts several months and serves as a transitional phase before the turtles move into the oceanic juvenile stage (Conant et al., 2009).

4. Juvenile Stage

Juveniles enter the oceanic stage, where they remain for several years. This stage can last between 7 to 24 years, depending on several factors, including food availability and

environmental conditions. During this time, juveniles grow significantly, eventually reach a size that allow them to move into neritic zone where they continue to mature. The timing of this transition varies by region; juveniles in the Mediterranean enter neritic habitats at smaller sizes compared to those in other regions such as Japan and Australia. These last two stages are also known as "The Lost Years" since there are still many unknowns about what happened during this period (Conant et al., 2009).

5. Adult Stage

As they reach maturity, they settle into neritic habitats where they feed, migrate, and breed. Some adults may periodically move between neritic and oceanic zones, but their primary foraging grounds are in coastal areas. Females exhibit site fidelity, often returning to the same beaches to nest each season, this is referred to as philopatry. The adult stage, particularly for females, can last for decades, and reproductive longevity has been documented to extend up to 25 years. The genetic differentiation between rookeries with nuclear markers suggest that both males and females need to show philopatric behavior (Clusa et al., 2018; Conant et al., 2009)

In sum, the loggerhead turtleś life cycle is characterized by distinct stages that involve critical migrations between different marine environments. These stages ensure their survival and reproductive success across diverse habitats, highlighting the importance of protecting both coastal and oceanic ecosystems.

1.3 Loggerhead turtles and climate change in the Mediterranean Sea

According to the IUCN, the loggerhead turtle is listed as Vulnerable at a global level (Casale, 2017) and as of Least Concern at the Mediterranean subpopulation level (Casale & Mariani, 2014). Despite been classified in this not critical category, the species has been included in international frameworks of protection and recommendations to

fisheries managers regarding the incidental catch of organisms in the Mediterranean Sea, in addition to having guidelines for assessing conservation actions to mitigate threats and allow the turtles to establish new rookeries (Hochscheid et al., 2022)

The life cycle of the Loggerhead sea turtle is composed by several factors that make them vulnerable to climate change. This species could adapt to increasing sand temperatures through changes in reproductive phenology, nest depth, and nest site selection. However, evolution may not be fast enough to keep pace with global warming. The species imperfect philopatry suggests that females may switch beaches even within a single nesting season, which could be a rapid response to global warming (Barbanti et al., 2022; Carreras et al., 2018). This shift to beaches with optimal temperatures is crucial, as overly warm beaches result in high embryonic mortality and lower recruitment of adults (Cardona et al., 2023).

The colonization of new nesting areas beyond the current breeding area is a process that has occurred in the past (Wallace et al., 2023). Turtle hatchlings passively disperse by ocean currents, establishing feeding and juvenile development areas far from their natal beaches, but within a short distance of other coastal areas. As they grow, juveniles migrate to areas close to their beaches of origin, although some turtles remain in these development areas until adulthood. If thermal conditions allow the development of gonads and embryos, these adults may nest on nearby beaches, which could explain nesting outside the usual range and could form new populations (Carreras et al., 2018).

Historically, loggerhead nesting in the Mediterranean has been limited to the eastern basin (Casale et al., 2018), with low levels of nesting in Calabria (Italy) (Figure 1). Genetics suggest at least two independent colonization events in the region where Calabria could represent a more recent colonization event (Clusa et al., 2013). In the western Mediterranean nesting has been sporadic since 1870 (Salvador et al., 1974).

Low sand temperatures prevented regular nesting during the 20th century (Pike, 2014) and probably before. In Spain, the detection of nesting events (false tracks, nests and hatchlings) has increased steadily since 2001 and this increase in nesting events has recently been correlated with increasing SST (Cardona et al., 2023).

Models based on climatic variables suggested that the Spanish Mediterranean coasts and the entire European coast of the western Mediterranean would be suitable for regular nesting of loggerhead turtles in 2020 (Pike, 2014). The increasing detection of nests in Spain in the last 20 years seems to confirm this prediction. Furthermore, genetic analysis has revealed a mixture of progenitors of Mediterranean and Atlantic origins, ruling out that these nesting events are remnants of a nearly extinct population (Carreras et al., 2018). The evidence supports the hypothesis that loggerhead turtles are colonizing the western Mediterranean due to global warming.

1.4 Genetics of the loggerhead sea turtle

Much of the genetics on *C. caretta* has taken advantage of maternal philopatry, so mitochondrial markers have been widely used (Shamblin et al., 2014). Nonetheless, the species has also been explored using nuclear microsatellites (Carreras et al., 2006; Clusa et al., 2018) and, more recently, SNPs (Barbanti et al., 2022).

1.4.1 Nesting genetics in the North Atlantic

Genetic studies of *Caretta caretta* nesting in the North Atlantic have revealed significant population structuring, driven by the species' philopatry, leading to clear genetic differentiation among nesting populations. In this regard, mtDNA analyses have shown that loggerhead populations in the southeastern US, one of the largest nesting areas, are genetically distinct from those in the Cape Verde Archipelago, a major nesting site in the eastern Atlantic (Bowen & Karl, 2007; Shamblin et al., 2014). Significant connectivity between nesting and foraging areas has subsequently been observed across

the Atlantic, including those in the Mediterranean, despite genetic differentiation among colonies. This mixing, which occurs at different stages of the life cycle, underlines the complexity of managing *C. caretta* populations (Monzón-Argüello et al., 2009; Shamblin et al., 2014).

1.4.2 Nesting genetics in the Mediterranean

In the Mediterranean Sea, the loggerhead turtle is the most widely distributed marine turtle species, with the main nesting colonies in Greece, Turkey, Cyprus and Libya (SPA/RAC-UNEP/MAP, 2021). Maternal and paternal philopatry has generated genetic differentiation between the Mediterranean and Atlantic subpopulations (Carreras et al., 2011; Shamblin et al., 2014), creating an independent RMU (Wallace et al., 2023). Currently, sporadic nests have been recorded on the western Mediterranean coasts, suggesting an emerging population associated with rising sea surface temperatures (SST), making this area a viable habitat for nesting females (Fig.2 (Maffucci et al., 2006)). Moreover, the contribution of Atlantic colonies to the Mediterranean rookeries remains poorly studied (Carreras et al., 2011).

Assesing individuals in the foraging grounds

During juvenile stages, individuals of *Caretta caretta* from different nesting sites move towards feeding areas, guided by surface currents that take them away from their breeding grounds. Upon reaching maturity, they return to their native coast to breed (Bowen & Karl, 2007). The contribution of various nesting sites to any foraging ground has been determined by mixed stock analyses that considers both the population size at the site of origin, its genetic variation and may be affected by the prevailing current patterns that connect these regions. Due to the complexity of this behaviour, individual assessment of the origin of individuals is crucial to identify populations that might be at risk outside their sites of origin.

Foraging grounds in the Mediterranean host both Mediterranean and Atlantic individuals (Carreras et al., 2006). This is because the North Atlantic, which contains the largest concentration of nesting in the region, is connected to European coasts through the Gulf Stream (Bolten et al., 1998). Although Atlantic loggerhead turtles enter the Mediterranean to feed, they rarely breed there, leaving it at juvenile stages when they become strong enough to swim against the currents of the Strait of Gibraltar (Eckert et al., 2008; Revelles et al., 2007). Therefore, the relative abundance of juveniles of Atlantic origin decreases towards the eastern Mediterranean although its finescale distribution remains elusive (Carreras et al., 2006; Clusa et al., 2014).

Assessing the distribution of loggerhead turtles in foraging areas has involved the use of several methods, such as satellite tracking, capture-mark-recapture and satellite telemetry (Casale et al., 2018). However, these methods have limitations and have been mostly restricted to adults. Genetics has leveraged maternal philopatry to identify genetic groups based on mtDNA analysis to understand population mobility and the relative contributions of different colonies to specific foraging areas are typically determined using mixed stock analysis (MSA). Conventionally, a short fragment of non-coding mitochondrial DNA (mtDNA) was used as a genetic marker for MSA (Carreras et al., 2011; Saied et al., 2012). Subsequently, sequencing of a longer segment of the mitochondrial control region significantly improved the resolution of the assignment (Abreu-Grobois et al., 2006; Clusa et al., 2014; Shamblin et al., 2011). Other markers to explore the genetic structure and origin of juvenile loggerhead turtles have been nuclear microsatellites (Carreras et al., 2011; Clusa et al., 2016; Yilmaz et al., 2011), allowing for individual based assignments. However, issues such as calibration problems, interpretation bias, low population differentiation based on the markers used, and presence of null alleles can introduce biasses and affect the robustness of the assignments (Abdul-Muneer, 2014).

Despite efforts, a precise individual assignment at the population level that allows identifying the origin of juveniles from several nesting populations in the Mediterranean feeding areas remains elusive. Recent advances in genomics offer high-resolution tools for these studies, in this sense, the 2b-RAD technique has been used to genotype 11 nesting localities in the eastern Mediterranean (Barbanti et al., -, in prep), which provides the opportunity to develop a baseline that allows finally assigning individuals at the global RMU level, RMU within the Mediterranean and even at the local level.

1.5 Evolution in action: Emerging areas

Rising ocean temperatures due to climate change are already having an impact on the ecological processes of marine species, so many species are expected to shift their distribution to remain in their suitable thermal habitat. Sea turtles are particularly vulnerable to climate change, as their reproductive success is closely linked to incubation temperatures. Warmer temperatures can reduce hatching success and lead to increased feminization of embryos. The ability of turtles to cope with projected increases in environmental temperatures will depend on their ability to adapt to changing climate regimes. We called this: *Evolution in action*, as we may be witnessing how species have coped with a changing environment in the past. The difference is that environmental change is now occurring at an unprecedented speed, and the threat is not giving species time to establish themselves in the new suitable areas and remain in the ecosystems. It is therefore crucial that these new distribution areas, such as emerging areas, design measures that guarantee an adequate environment for the species to thrive (Abella Perez et al., 2016).

1.6 Genomics of Loggerhead turtles

The study of the population genetics of this species has always been associated with the challenge of dealing with a non-model species due to its long-time generation and its large genome. *C. caretta* has a total of 28 pairs of chromosomes: 12 macrochromo-

somes and 16 microchromosomes (Fig 7) (Kamezaki, 1989).

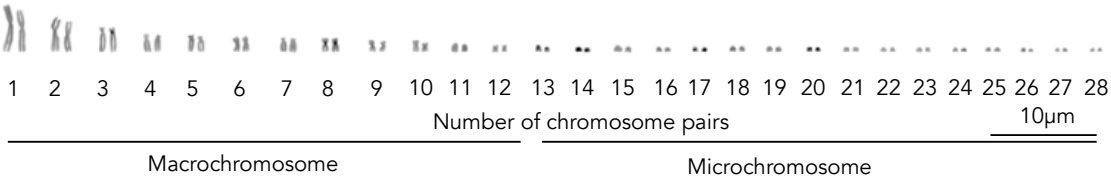

Figure 7: Karyotype of the loggerhead sea turtle (*C. caretta*). The karyotype is composed of a total of 56 chromosomes, organized in 28 pairs. Of these, the first 12 pairs correspond to macrochromosomes, the remaining 16 pairs are microchromosomes. Picture from Machado *et al* (Machado et al., 2020).

Population genetics studies were also limited by a small number of molecular markers, until the arrival of population genomics, which revolutionized the scope of each study by going from tens to thousands of markers. Recent advancements in genomics offer high-resolution tools for these studies, in this sense, the 2b-RAD technique has numerous advantages for studying non-model species. This method is a cost-effective substitute to other reduced-representation genotyping methods and has proven particularly effective in species with large genomes, such as the loggerhead sea turtle with an approximate size of 2.3 gigabases (Gb) (Chang et al., 2023). Moreover, 2b-RAD facilitates population genetic analyses, providing detailed ecological and evolutionary insights that were previously challenging to achieve (Barbanti et al., 2020).

The recent sequencing of loggerhead turtle (*Caretta caretta*) individuals from nine localities in the Eastern Mediterranean nesting area (Barbanti et al., -) offers significant potential to advance research on feeding and breeding grounds. These genomic data provide a powerful tool for genomic studies, since it significantly demonstrates the effectiveness of the representation of the genomics of the species, since it is observed that the distribution of the SNPs is correlated with the size of the chromosomes throughout the genome of the species (Figure 8).

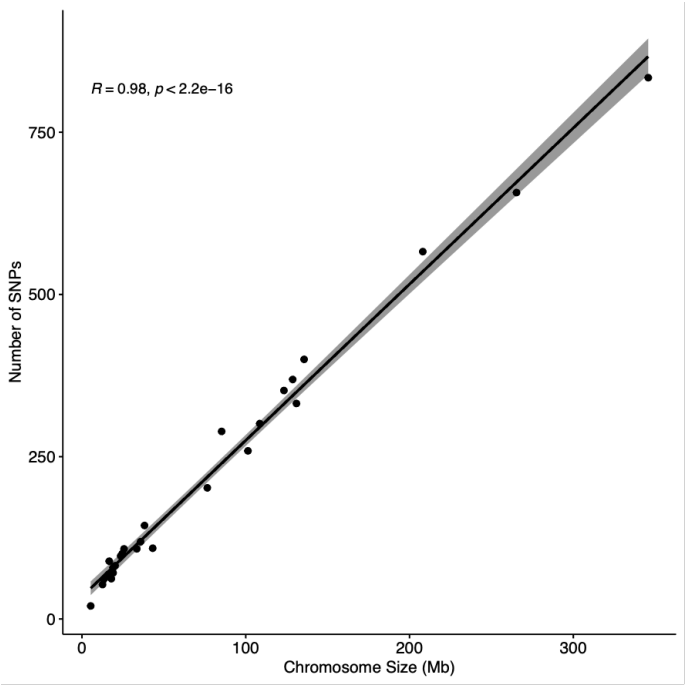

Figure 8: Distribution of SNPs in the loggerhead genome. Correlation between the number of SNPs on each one of the 28 loggerhead genome chromosomes and the chromosome size. R and p-values of the Spearman correlation are included.

The genomics of the species allows individuals to be accurately assigned to populations and increasing the understanding of population structure and connectivity. By tracing genetic links between foraging and nesting grounds, it would be possible to refine conservation strategies and implement more targeted protection measures. These efforts will be crucial to addressing the ecological and migratory patterns of loggerhead turtles across the Mediterranean and Atlantic, thereby improving their long-term conservation.

02

OBJECTIVES

2. OBJECTIVES

The present doctoral thesis has as its main objective to study the loggerhead sea turtle at different stages of its life cycle to answer unresolved questions about population structuring, migration and adaptation. For this reason, we applied genomic tools to evaluate the nesting populations and foraging aggregations of the loggerhead turtle (*Caretta caretta*) and new emerging nesting areas in the context of global warming.

- Evaluate the genomic diversity and hierarchical population structure of the nesting populations in the North- West Atlantic, North-East Atlantic, and Mediterranean Regional Management Units
- Develop a methodology for genomic based individual assignments (IA) of turtles to their origin nesting populations.
- Asses the origin of the individuals in foraging grounds aggregation in the Mediterranean at regional and local level
- Design a guideline for the methodology developed for use in highly migratory non-model species
- Characterize the genomic composition of sporadic nests laid on the Mediterranean coast of Spain from 2016 to 2019 to assess the possible causes of the increase in the number of colonizing events.
- Evaluate the origin of these nesting events to assess the origin of the colonization and to detect potential processes of admixture among individuals from different origins.

Thesis Organization

This thesis has focused on the study of the loggerhead sea turtle (*Caretta caretta*) throughout different stages of its life cycle. To do so, we have used the 2bRAD genomics technique, taking advantage of the reduced representation genotyping to study non-model species. The research conducted in this thesis is presented in three independent chapters and the discussion of their global results integrated in the general discussion.

Chapter 3.1: "Genomic individual assignment on highly migratory species: baseline and application on the loggerhead turtle"
We have built a genomic baseline that integrates localities from regular nesting areas of the species in the North Atlantic (both West and East) and Mediterranean Sea, evaluated genetic differentiation between these three areas, and subsequently carried out the individual assignment of juvenile turtles sampled in Mediterranean foraging grounds with the aim of identifying their origin

Chapter 3.2 : "New colonisers drive the increase of the emerging loggerhead turtle nesting in Western Mediterranean"
Nesting events have occurred and increased in the Western Mediterranean in recent decades. In this chapter, we have genomically analyzed the nesting events that occurred in Spain from 2016 to 2019. We also present a colonization model and a series of hypotheses that aim to provide an answer to the origin of this phenomenon.

Chapter 3.3 : "Individual genomic assignment of loggerhead sea turtles in the emerging nests in Spain"
Finally, we present the combination of both chapters in this thesis. We have used the genomic baseline constructed in Chapter 3.1 to study the origin of breeders in the nests analyzed in Chapter 3.2 (2016-2019) and report the assignment probability hatchlings reflecting the origin of the breeders at the regional and subregional level.

03 CHAPTERS

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CHAPTER 3.1

GENOMIC INDIVIDUAL ASSIGNMENT ON HIGHLY MIGRATORY SPECIES: BASELINE AND APPLICATION ON THE LOGGERHEAD TURTLE

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ABSTRACT

Genomic techniques are becoming widely used in conservation offering an unprecedented resolution to evaluate the behavior, reproductive success and impact of different threats on endangered species. In the case of sea turtles, the natal origin of juveniles in foraging areas or breeders on emerging nesting beaches needs to be assessed to make scientifically informed management decisions. Furthermore, genomic reduction techniques are frequently used to optimize costs in species with large genome sizes, although it is fundamental to apply the same methodology across regions for cross-comparisons. However, to assign individuals it is crucial to build a genomic baseline with data from known populations, represented by regular nesting areas in the case of sea turtles from where the individuals could have originated. To build a robust baseline for the analysis of juvenile loggerhead turtles in Mediterranean foraging grounds, we analyzed with 2bRAD 278 individuals from 3 regional management units (RMU): the Mediterranean RMU (MED) represented by individuals from 11 nesting beaches, the North West Atlantic RMU (NWA) represented by individuals from 2 nesting beaches, and the North East Atlantic RMU (NEA) represented by individuals of 1 nesting beach. Additionally, we genotyped with the same methodology 103 individuals from 4 foraging areas along the Mediterranean Sea to test the potential of genomics for individual assignments in marine turtles: Catalan region (CAT), Lampedusa (LAM), Eastern Aegean Sea (EAS) and Western Aegean Sea (WAS). First, we carried out a genetic structure analysis with all the baseline individuals using 5,308 SNPs, genotyped in more than 70% of the samples, and identified high genetic differentiation between the three RMUs. Secondly, we performed a hierarchical analysis with only the samples from the Mediterranean nesting grounds and identified three groups Greece, Levantine and Sirte nesting areas with a clear differentiation between them, thus considered as SubRMUs within the Mediterranean. To test the effectiveness of this baseline for individual assignments, we followed a hierarchical approach from a global perspective (including the baseline of the three RMU), to a more regional perspective (including only the Mediterranean nesting beaches as baseline) and successfully identified the origin of each of the 103 unknown

individuals collected in different foraging grounds to the RMU level. When analyzing at sub-regional level the origin of the individuals assigned to the Mediterranean RMU, the individuals from Levantine were more prevalent in general with some individuals having mixed assignments indicating the existence of breeding between sub-regions. Our methodology allowed us to perform individual assignments at RMU and SubRMU level on all samples introducing new perspectives to the research and conservation of sea turtles. By using this strategy, it is possible to effectively identify the populations impacted by threats outside their nesting areas, or study turtles from a specific origin found in mixed foraging areas.

Key words: *Caretta*, *Loggerhead*, *Genomics*, *Baseline*, *Individual assignments*

INTRODUCTION

Distribution and dispersal of many marine mega vertebrate taxa involve large spatial and temporal scales. These highly migratory species have wide geographic distributions and undertake cyclical movements between distinct geographic areas for foraging or reproduction, allowing to exploit feeding resources in areas unsuitable for breeding (Lascelles et al., 2014). Consequently, many anthropogenic threats may affect their populations at various stages of these movements, including fisheries interaction, pollution, habitat degradation or interaction with marine traffic (Cardona et al., 2005; Clusa et al., 2016). Effective conservation is only possible by understanding the distribution of these migratory species and the interplay between the breeding areas and distant anthropogenic threats at foraging areas.

Sea turtles are an iconic example of a highly migratory marine species, as they can perform transoceanic migrations along established routes throughout their life cycle from breeding to foraging areas (Bowen et al., 1995; Hays et al., 2002). However, these species have philopatric behavior that contribute to the genetic structuring of breeding populations, meaning that adult female turtles return to nest in their areas of origin despite being highly migratory (Amorocho et al., 2012; Carreras et al., 2007; Leroux et al., 2012). Genetic studies have recently suggested that both sexes need to show philopatric behavior to explain the genetic differentiation found with nuclear markers (Clusa et al., 2018, Barbanti et al., -, in prep). The combination of a deep genetic structuring, favored by philopatry, with long migrations across oceans raised the need to define a new unit for conservation above the population level but below the species level, the Regional Management Units (RMUs) (Wallace et al., 2010). The RMUs combine multiple sources of information (nesting sites, population abundances and trends, population genetics, and satellite telemetry) to define a group of populations that share migration routes, foraging areas and, consequently, are exposed to similar threats and share evolutionary trajectories. Therefore, understanding the patterns of widespread dispersal of hatchlings from breeding sites in the RMUs to their develop-

mental and foraging habitats, crossing political and ecological boundaries (Bolten et al., 2003; Bowen & Karl, 2007; Luschi & Casale, 2014) is key to undertake correct management and conservation actions. However, this is not an easy task, as sometimes individuals from different RMUs are known to share some foraging areas, adding a new level of complexity as a mixed aggregation may be used not only by genetically different populations but also by different RMUs (Wallace et al., 2023).

The foraging areas of the loggerhead sea turtle (*Caretta caretta*) in the Mediterranean provide one of the best examples of the challenges posed by individual assignments in highly migratory species. Previous studies indicated that the foraging areas in the Mediterranean host turtles from up to three different RMUs: the Mediterranean RMU (MED RMU), the North West Atlantic RMU (NWA RMU) and the North East Atlantic RMU (NEA RMU) (Carreras et al., 2011; Clusa et al., 2014; Monzón-Argüello et al., 2009). Furthermore, although the IUCN Red List globally classifies loggerhead turtles as "Vulnerable" (Casale & Tucker, 2017) , this category varies by RMU due to the different threat intensity they face, including illegal harvesting, habitat degradation, pollution, fisheries bycatch, and climate change (Casale et al., 2018). Particularly, the Mediterranean and North West Atlantic RMUs have a different category (Least Concern) than the North East Atlantic RMU (Endangered). Consequently, knowing the RMU of origin is paramount to assess the importance of the mortality of juveniles in Mediterranean foraging areas on their natal beaches. A similar situation arises when looking into the detail of the Mediterranan RMU. Recent studies revealed that the Mediterranean nesting populations are genetically differentiated (Clusa et al., 2018), although with a clear intermediate level of structuring in three genetic clusters when assessed at the individual and not the population level (Barbanti et al., -, in prep). These three groups cluster in one all the populations of Greece, in another group the populations of the Levantine region (Turkey, Lebanon, Israel and those in the island of Cyprus) and the third cluster individuals from Libya. These three genomic clusters align with the predicted patterns of dispersion of hatchlings and have been proposed as sub regional

management units (SubRMU, Casale and Mariani 2014). Consequently, the different layers of the hierarchical structuring of the species (from populations to SubRMU and RMU) make the assignment of individuals at sea even more challenging.

The assignment of animals at sea to their natal areas in marine turtles has been primarily performed using genetics (Komoroske et al., 2017). To do so, the genetic profiles of the individuals of unknown origin are compared to a dataset of genotypic information gathered from the potential nesting areas of origin, commonly referred as 'baseline' or 'stocks'. The first approaches leveraged maternal philopatry to breeding areas to define genetic clusters using mitochondrial DNA (mtDNA) markers, for understanding population structure and mobility using mixed-stock analysis (MSA). This analysis allows to infer the percentage of individuals coming from the different populations of the baseline (Grant et al., 1980) but does not assess the origin of each single individual independently. MSA has been frequently based on a fragment of the D-loop of the mtDNA. Initially, a short fragment of the D-loop was used to assess the genetic structure of the nesting populations (Carreras et al., 2007; Laurent et al., 1998; Saied et al., 2012) and combined with data of foraging areas for MSA (Carreras et al., 2006; Maffucci et al., 2006). However, the presence of highly abundant haplotypes shared among the potential source populations undermined the detection of genetic differentiation in some cases and produced stock assessments with large confidence intervals. The sequencing of a longer segment of the same region significantly enhanced the genetic resolution of the baseline (Abreu-Grobois et al., 2006; Clusa et al., 2013; Monzón-Argüello et al., 2010; Shamblin et al., 2014) and slightly improved MSA (Clusa et al., 2014). However, individual assignments (IAs), that is the ability to assign a specific individual to their natal origin, were not yet feasible due to the overdominance of common haplotypes across the baseline populations. The use of nuclear microsatellites added a new layer of information on the population structure of the baseline (Carreras et al., 2007; Clusa et al., 2018) and allowed for the first time IAs on marine turtles (Carreras et al., 2011; Clusa et al., 2016). However, the low number of markers was found to be fundamental

for a correct identification of population structure and limited the individual assignment to the RMU level with a significant portion of the individuals being unassigned.

Recent advancements in genomics offer high-resolution tools for conservation genomics, due to the large number of markers genotyped at many individuals. In this sense, the 2b-RAD technique has proven to be effective in species with large genomes, such as the loggerhead sea turtle (Barbanti et al., 2020, Chapter 3.2.) The recent study on the eastern Mediterranean nesting populations using the 2b-RAD technique (Barbanti et al., -, in prep), detected unprecedented levels of genetic differentiation among populations and confirmed the existence of an intermediate level of structuring within the Mediterranean RMU in three SubRMUs. This study showcased the potential of genomics to build a robust baseline and opened the possibility of applying this approach to perform IAs in the Mediterranean foraging areas thereby providing an opportunity to evaluate capabilities of next-generation sequencing, improving scientifically based information for managing species conservation.

The aim of this study is to build a genomic baseline of the Atlantic-Mediterranean loggerhead turtles and test its utility to perform individual assignments in juveniles from four Mediterranean foraging grounds to their areas of origin. We first assessed the genomic variability and genetic differentiation of 278 individuals sampled at 11 nesting populations from three regional management units: Mediterranean, North West Atlantic and North East Atlantic. We second develop a methodology to perform Individual Assignments by testing different genomic Baselinesfollowing a hierarchical approach considering the three RMUs or only the Mediterranean RMU. Third, we use the methodology proposed here to identify the natal origins of 103 juvenile loggerhead turtles sampled in four foraging regions in the Mediterranean Sea by doing individual assignments. Finally, we propose a guide of best practices to perform individual assignment of loggerhead turtles using genomic baselines that can also be implemented in other highly migratory species.

METHODOLOGY

Sampling

To build a genomic baseline of Atlantic-Mediterranean loggerhead turtles, we analyzed in total 278 individuals in nesting populations (Table 1) known to belong to three RMUs (Fig 1): 15 individuals from the North West Atlantic (NWA), 25 from the North East Atlantic (NEA) and 238 from the Mediterranean (MED), the latter previously published by Barbanti et al., -, in prep. The samples from the NWA RMU (Table 1) comprise 12 individuals from Quintana Roo (QRO, Mexico) and 3 individuals from Grand Cayman (CAY, Cayman Islands). The 25 individuals from the NEA RMU were all from Boa Vista (BOA, Cape Verde). Finally, the samples from the MED RMU were from 11 nesting areas: 22 individuals from Sirte (SIR, Libya), 20 from Israel (ISR), 19 from El Mansouri (LEB, Lebanon), 25 from Alagadi (ALA, Cyprus), 25 from Akamas (AKA, Cyprus), 23 from Belek (BEL, Turkey), 24 from Dalyan (DAL, Turkey), 17 from Messara (MES, Crete, Greece), 22 from Rethymno (RET, Crete, Greece), 25 from Kyparissia (KYP, Greece), and 16 from Zakynthos (ZAK, Greece). To avoid pseudoreplication (e.g., sampling hatchlings from nests of the same female), dead hatchlings were collected either from nests of known-identity females (through tagging) or from nests laid within a 14-day window, or in two consecutive nesting seasons, as in previous studies (Carreras et al., 2007). One hatchling per nest was sampled. Additionally, we collected 103 samples (Table 1) from 4 Foraging Grounds (FGR) in the Mediterranean (Fig 1): 26 samples from the Catalan Coast (CAT), 28 samples from Lampedusa (LAM), 23 from the West Aegean (WAS) and 26 from the East Aegean (EAS). Samples from the FGR were from animals that arrived at rescue centers in the area. Skin samples were obtained from dead stranded animals while blood samples were obtained from living animals following the procedures of the rescue centers. All tissue and blood samples were fixed and stored in 96% ethanol at -20°C until DNA extraction.

Table 1: Information on the *C. caretta* individuals genotyped from Nesting locations and Foraging regions (Group). Regional Management Unit (RMU) of the sampled location and countryarea (Location), location acronym used (Acronym), latitude and longitude of the nesting location and geographic range of the samples in each foraging ground. Number of individuals analyzed in each location (n), mean number of reads obtained per individual and mean observed heterozygosity (Ho) calculated considering the 5308 polymorphic SNPs of the RMU Baseline dataset. The three RMUs stand for North West Atlantic (NWA), North East Atlantic (NEA), Mediterranean (MED), while Unknown (UNK) correspond to individuals of unknown origin sampled in the foraging grounds to be assessed. Raw data of these samples was obtained from (Barbanti et al., -, in prep)

Group	RMU	Location	Acronym	Latitude	Longitude	n	Mean Number of Raw Reads	Ho
Nesting	NWA	Quintana Roo (Mexico)	QRO	20,33	-87,35	12	4815187	0.2034
Nesting	NWA	Cayman (Cayman Island)	CAY	19,28	-81,2	3	1949924	0.2123
Nesting	NEA	Boa Vista (Cabo Verde)	BOA	16,04	-22,69	25	5777845	0.2048
Nesting	MED	Sirte (Lybia)	SIR*	31,31	16,6	22	5221174	0.2155
Nesting	MED	Israel (Israel)	ISR*	32,35	34,64	20	5657554	0.2151
Nesting	MED	Lebanon (Lebanon)	LEB*	33,24	35,06	19	4166352	0.2150
Nesting	MED	Alagadi (Cyprus)	ALA*	35,2	33,29	25	5162231	0.2143
Nesting	MED	Akamas (Cyprus)	AKA*	35,04	32,26	25	5522622	0.2118
Nesting	MED	Belek (Turkey)	BEL*	36,82	31,06	23	4584541	0.2122
Nesting	MED	Dalyan (Turkey)	DAL*	36,78	28,58	24	4501977	0.2137
Nesting	MED	Messara (Greece)	MES*	34,92	24,66	17	5703729	0.2165
Nesting	MED	Rethymno (Greece)	RET*	35,38	24,47	22	4684613	0.2147
Nesting	MED	Kyparissia (Greece)	KYP*	37,26	21,66	25	4658342	0.2068
Nesting	MED	Zakynthos (Greece)	ZAK*	37,72	20,89	16	3370878	0.2218
Foraging	UNK	Catalan Coast (Spain)	CAT	40,61 - 42,36	0,59 - 3,16	26	4769814	0.2344
Foraging	UNK	Lampedusa (Italy)	LAM	35,52 - 35,50	12,51 - 12,63	28	4351789	0.2194
Foraging	UNK	West Aegean Sea	WAS	37,04 - 40,92	22,93 - 26,97	23	3242724	0.2263
Foraging	UNK	East Aegean Sea	EAS	37,34 - 38,67	26,75 - 27,32	26	4060632	0.2170

Laboratory procedures

Genomic DNA was extracted using the Puregen Kit (Qiagen) following the manufacturer's instructions and resuspended in 25 µl of Elution Buffer. The DNA concentration and quality was measured with Nanodrop. We constructed individual libraries for the 143 individuals sampled in this study (Table 1) using the previously published protocols (Barbanti et al., 2020, Chapter 3.2, (Barbanti et al., -, in prep)). In brief, we digested

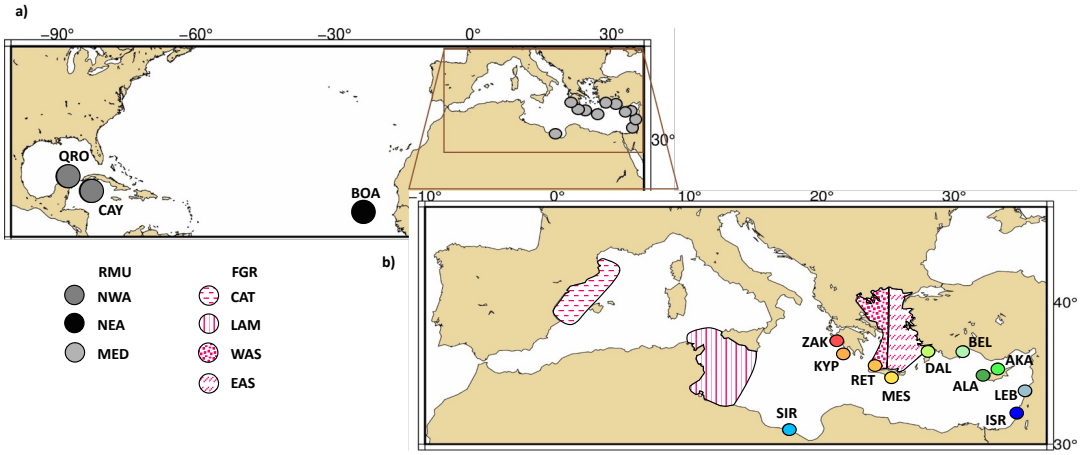

Figure 1: Sampling locations of the nesting populations for the baseline and the foraging grounds. Map with all sampling locations of the study. a) Baseline samples were obtained from nesting areas from three Regional Management Units (RMU) to build a genomic baseline for individual assignments. Sampled RMUs are defined in the literature (Wallace et al., 2023): North West Atlantic (dark gray, NWA), North East Atlantic (black, NEA) and Mediterranean (light gray, MED). b) Samples within the Mediterranean include data from nesting locations to be used as a baseline at Subregional Management Unit level obtained from the literature (Barbanti et al., -, in prep): Zakynthos (ZAK; N=16), Kyparissia (KYP; N=25), Rethymno (RET; N=22), Messara (MES; N=17), Dalyan (DAL; N=24), Belek (BEL; N=23), Akamas (AKA; N=25), Alagadi (ALA; N=25), Lebanon (LEB; N=19), Israel (ISR; N=20) and Sirte (SIR; N=22). Texture frames indicate the foraging grounds of individuals from unknown origin samples in this study: Catalan coast (CAT; N=26), Lampedusa (LAM; N=28), Western Aegean Sea (WAS; N=23) and Eastern Aegean Sea (EAS; N=26). Both maps were created using the free software MAPTOOL (SEATURTLE.ORG Maptool. 2002. (Wessel et al., 2013).

180 ng of DNA (≈ 40ng/µl) with the AlfI enzyme and ligated the fragments with base selective-adaptors (5'-WN-3') that reduce the number of analysed markers without compromising genetic differentiation (Galià-Camps et al., 2022). The quality of the amplified fragment was verified in a 1.8% agarose gel. The successful amplified product was purified using magnetic beads to remove primers and fragments shorter than 165 bp. The DNA concentration of the purified libraries was measured with the Quant-iTTM Picogreen dsDNA Assay Kit (Thermo Fisher Scientific) and pooled considering the same type of index and calculating 180 ng of DNA per library. During the construction of these libraries the methodology was modified, changing from single index to double index, the affected the size of the amplified fragment but did not affect the library construction or bioinformatic analysis. The pool was sequenced with a HiSeq 2500 Ilumina for single index and NextSeq 500 for dual index at the Centre for Genomic Regulation (CRG).

Data genotyping and filtering

We processed the 2bRAD sequences using customized scripts, trimming the raw sequences to remove ligation adaptors and cutting all the fragments to the same length (34 bp) (https://github.com/EvolutionaryGenetics-UB-CEAB). We mapped the trimmed sequences to the published reference genome of the loggerhead turtle (GenBank accession GCA_023653815.1, (Chang et al., 2023)) using Hisat2-2.2.183 (Kim et al., 2019), to identify polymorphic nucleotides (SNPs) with BCFtools (Li 2011), and individual genotypes were outputted as SNPs in a VCF file. Two different strategies were followed to evaluate the impact of the number of samples in the genotyping step for the unknown individuals. In the first case, named Joint Genotyping (JG), we genotyped all the samples of the study simultaneously to generate a single vcf file with all the genotypes and, from the resulting file, we separated the baseline data from the unknown data using the –keep function of VCFtools (Danecek 2011). In the second case, named Non-Joint Genotyping (NJG), the unknown samples were genotyped independently. In the two strategies, we filtered the baseline genotypes using VCFtools, by removing individual genotypes based on less than five reads and loci with a mean depth above 50 (which corresponds to the upper whisker defined as 1.5 times the interquartile range from the data). Additionally, filtering was performed on later steps depending on the datasets generated from the initial files (see details below). The final set of SNPs obtained from the different Baseline datasets was used to extract the same markers from the file with the genotypes of the unknown individuals, using the '–snps' function in VCFtools. This step was done for both the JG and NJG file of unknown individuals.

In this study, we followed a hierarchical approach to assess individual assignments that can be widely used for assignation from RMU to SubRMU levels. Consequently, we generated specific baseline datasets for each one of the levels of the genetic structuring of the loggerhead in the Atlantic and Mediterranean. All these datasets were built using the vcf files generated from both the joint genotyping and non-joint genotyping strategies to evaluate which genotyping strategy yields more polymorphic markers. For this, at Regional Managment Unit level, we first built a RMU Baseline with all the 278

samples from NWA, NEA and MED. Additionally to evaluate the effect of having large differences in the number of sampling individuals in the three RMUs an adjusted data set was built by reducing the number of samples from the Mediterranean RMU. Thus, the RMU Baseline-R (baseline with the number of individuals reduced to reach more similar numbers in each RMU) was composed of 84 samples including all NWA (n=15) and NEA (n=25) individuals, and 4 individuals from each location in MED (n=44, See Individuals.txt). Finally, to assign at the SubRMU level within the Mediterranean, the MED Baseline data set was built considering all the 238 samples from MED with the 3 SubRMUs detected in previous studies (Barbanti et al., -, in prep) and confirmed in the present work (see results): Greek SubRMU (Zakynthos, Kyparissia , Rethymno and Messara), Levantine SubRMU (Dalyan , Belek, Akamas, Alagadi, Lebanon and Israel) and Sirte SubRMU (Libya). In each baseline data set, loci present in less than 70% of the individuals were removed using the –max-missing function of VCFtools and only polymorphic loci were retained by adjusting the minimum allele frequency, to retain a minimum of 5 alternative alleles per SNP, using the –maf function of the same program. After identifying the best strategy to retain the highest number of markers in the baseline and foraging grounds, for subsequent analyses only the vcf files with the best genotyping strategy (joint genotyping vs non joint genotyping) were used.

For the individuals in the foraging grounds, we used the vcf file with only the unknown individuals to be assigned with the same markers than the corresponding baseline dataset used to perform the individual assignments (RMU Baseline, RMU Baseline-R or MED Baseline), obtained as explained above. At RMU level, data sets with the 103 unknown samples from CAT, LAM, WAS and EAS were obtained with the same SNPs as in the RMU Baseline or the RMU Baseline-R. While, in a next step, at the Mediterranean level, we discarded the individuals assigned to the Atlantic and kept only the individuals assigned to the Mediterranean and selected the same SNPs retained in the MED Baseline.

Genomic diversity and structuring of the baseline samples

We used the SNPs retained in the RMU Baseline dataset to estimate the Observed Heterozygosity (Ho) using the function '–het' of VCFtools. The genetic differentiation among RMUs was calculated using pairwise FST distances calculated with Nei's estimator (Nei, 1987), and significance was assessed by 999 permutations to calculate the respective p-values in the R package 'hierfstat'. Heatmaps of genetic differentiation among RMUs were generated using the R package 'gplots' (Warnes et al., 2016). We also evaluated the genomic RMU differentiation with Discriminant Analysis of Principal Components (DAPC) using the 'adegenet' R package (Jombart, 2008) and following the method described by Jombart and Collins (2015). The the number of PCs to retain was assessed using the 'x.val' function and the optimal number of genetic clusters (K) was assessed based on the Bayesian Information Criterion value (BIC) using the 'find.cluster' function. In addition, we computed the Identity by State (IBS) individual pairwise distances and used them to perform a Multidimensional Scaling analysis (MDS) with PLINK vr. 1. 07 (Purcell et al., 2007) and represented the individual values for coordinates 1 and 2 represented.

Individual assignment of the unknown samples

Firstly, we evaluated the self-assignment probabilities of each baseline data set through two analyses. On one hand, we used the 'predict' function in R (Chambers & Hastie, 1992) considering the DAPC previously done through the 'adegenet' R package (Jombart, 2008). In addition, we used the R package assignPOP v1.1.4 (Chen et al., 2018) for testing the self-assignment accuracy of each baseline individual. All five models, provided by the 'assignPOP' v1.1.4 package, were tested (i.e., Support Vector Machine (SVM), Latent Dirichlet Allocation (LDA), NaiveBayes (NAIVE), decision Tree (TREE), and Random Forest (FOREST)) to identify which one better described the baseline data. A Monte Carlo cross-validation procedure was performed to cluster individuals into reference and test data sets using the 'assign.MC' function of assignPOP. Thirty iterations were run for each model, the proportion of individuals from each source population randomly allocated to the baseline data set was set to 0.5, 0.7, 0.9,

and the loci proportion was set to 0.1, 0.25, 0.5 and 1. The best model was used to estimate the self-assignment of each baseline data set using the 'assign.X' function. We first evaluated the self-assignment to the 3 RMUs: 1) NWA, 2) NEA and 3) MED of the individuals used in the RMU Baseline (n=279) and the RMU Baseline-R (n=84), independently. We also evaluated the assignment using only individuals of the MED Baseline. We considered 3 clusters as shown in DAPC (see Results): 1) GRE, 2) LEV and 3) SIR; and 2 clusters as in the MDS (See Results): 1) GRE and 2) LEV+SIR.

In all cases, two files with the same number of markers were used to perform the individual assignments: the baseline corresponding to the level of assignation to be tested (RMU Baseline, RMU Baseline-R or MED Baseline) and the file with the unknown samples to be assigned. The individuals from the foraging grounds were assigned by comparison to the different Baseline datasets using assignPOP. We carried out 10 tests to assign the individuals from the foraging grounds using the 'assign.X' function to each Baseline. We considered the mean and standard deviation of the values obtained in the 10 tests to evaluate possible biases among runs.

RESULTS

Effect of the genotyping strategy

All the 381 samples were successfully sequenced with a mean of 4,704,227 reads obtained per individual (SD±1,621,134; Table 1), and a mean mapping success of 93.37% against the reference genome (SD±5.97; Table S1). When building the baseline datasets, the two genotyping strategies yielded different results in terms of loci recovered when searching the presence of loci in the vcf file of foraging ground individuals found in the baseline. The Joint Genotyping (JG) strategy recovers consistently more polymorphic markers than the Non-Joint Genotyping (NJG) strategy. Specifically, in the RMU Baseline we recovered 5,308 SNPs, that were all recovered with the JG in the foraging ground dataset and only 5,076 SNPs in the unknown dataset when using the NJG (95.6% of the SNPs in the baseline). Similarly, in the RMU Baseline-R we

recovered 4,321 SNPs in the JG (100% of the baseline) and only 4,156 SNPs (96.2% of the baseline) in the NJG. Finally, in the MED Baseline, we retained 5,177 in the JG (100% of the baseline) and only 4,981 SNPs (96.2% of the baseline) in the NJG. Thus, we observed that by performing separately genotyping, only a total of 96% of the markers obtained with the baseline genotyping were recovered while all of them were recovered in the joint genotyping. Consequently, we selected the jointly genotyped SNP strategy for all the subsequent analyses.

With the 5,308 SNPs in the RMU Baseline, the mean observed heterozygosity by nesting locality was 0.2127 (SD± 0.004, Table 1). ZAK presented the highest observed heterozygosity (0.2218) and QRO presented the lowest (0.2034). When using the same panel of 5,308 SNPs in the unknown samples, the mean observed heterozygosity by foraging grounds was 0.2243 (SD± 0.004, Table 1), where CAT presented the highest observed heterozygosity (0.2344) and EAS the lowest (0.2170).

Genetic differentiation at the Regional Managment Unit level

All pairwise comparisons among RMUs were significantly different when using the RMU Baseline dataset (N=278). The comparison between the two Atlantic RMUs (NWA-NEA) presented the lowest FST value (FST =0.0226), and the greater and significant distance was observed between Atlantic RMU and MED (0.04815 ± 0.0057, mean ± SD) (Figure S1a; Table S2a).

A DAPC, retaining 20 components (n.pca), showed that the nesting individuals formed three clear clusters that corresponded to the three RMUs (Figure S1b). The first axis separated the individuals from the two Atlantic RMUs to those that belong to the Mediterranean RMU, while the second axis separated the individuals from the North West Atlantic (NWA) RMU from those nesting in the North East Atlantic (NEA) RMU (Figure S1b). Similar results were found with the MDS based on IBS individual pairwise

distances with the same dataset, revealing the same three clusters but with a tighter grouping of the individuals of the two Atlantic RMUs, not being differentiated by the second axis, and the Mediterranean individuals scattered along the second axis (Figure S1c).

To evaluate the impact of having different groups with large contrasting sampling sizes to infer individual based genomic differentiation, we performed all the previous analyses with the reduced dataset (RMU Baseline-R). The number of SNPs was smaller than in the RMU Baseline dataset (4321 SNPs) in accordance with the smaller number of individuals included in the dataset (n=84). However, the genetic structuring found was very similar, with all the RMU pairwise comparisons significantly different and similar FST values as when all the individuals were included (Figure S1a; Table S2b). In contrast with the RMU Baseline, the RMU Baseline-R clearly separated the individuals from the three RMUs, both with the DAPC (retaining 10 pcas) and with the MDS, clearly separating the individuals from the two Atlantic RMUs by the second axis (Figure 2b, c). Consequently, the uneven sampling size with a number of individuals from the MED RMU an order of magnitude higher than the other two RMUs reduced the ability to detect genetic differentiation between the two Atlantic RMUs in the MDS plot.

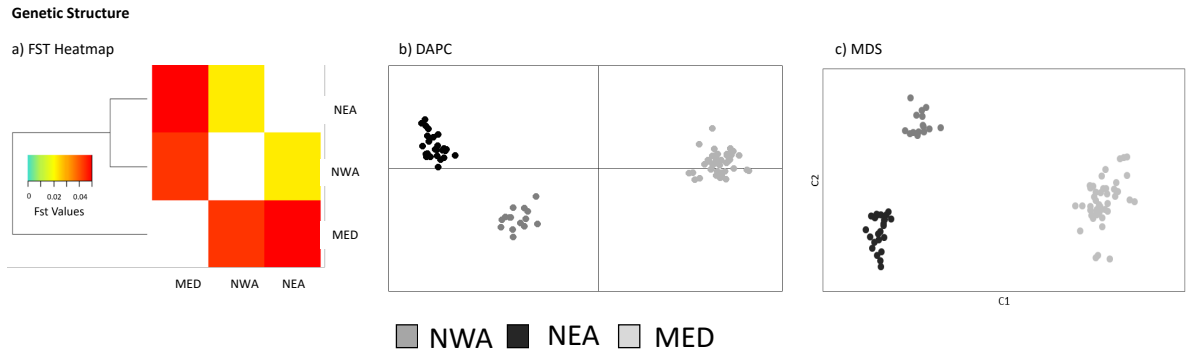

Figure 2: Genomic Structure of the reduced baseline to perform individual assignments at the RMU level. The reduced baseline (RMU Baseline-R dataset) was composed by a selection of samples from nesting populations (see text for details) (n=84, Table S2) from the 3 RMUs (NWA=North West Atlantic, NEA=North East Atlantic and MED= Mediterranean) and included a total of 4,321 filtered SNPs. a) Heatmap and dendrogram based on the FST pairwise RMU genetic distances (*Indicates significant differences); b) Discriminant analysis of principal components (DAPC); and c) Multi-Dimensional Scaling (MDS) analysis based on Identity by State (IBS) individual pairwise genetic distances. Each dot represents an individual color-coded according to RMU.

Genetic differentiation at the Mediterranean level

In a hierarchical approach, we next evaluated the genetic differentiation only using the individuals from the nesting locations in the Mediterranean following the same three analytical methodologies as for the genetic differentiation among RMUs. To do so, we used the MED Baseline with 238 individuals (Figure 1b) that retained 5,177 SNPs after filtering. The mean FST value between Mediterranean localities was 0.011995 (±0.027132, ± SD), where DAL and BEL were the localities that presented the lowest genetic differentiation (FST= 0.0004, Figure 3a, Table S2c), as found with 5998 SNPs by Barbanti et al., -, in prep, and ISR and ZAK showed the greatest genetic distance (FST= 0.0208). All values except six were significant after FDR correction (Figure 3a). Then, we plotted the population composition in genetic clusters. Firstly, in a DAPC we retained 20 components (n.pca) and 3 discriminants (n.da). The linear discriminant 1 clearly separated SIR (to the left) from the other MED localities that integrated a largest cluster (to the right). On the other side, in linear discriminant 2 the GRE populations make a cluster in more positive values (to the top), while the individuals of the LEV and SIR populations are distributed towards more negative values; thus, considering the two

axis the individuals of the three previously described SubRMU can be separated (Figure 3b). Lastly, in the MDS the analysis showed two fairly defined clusters that separated GRE (to the left) from LEV+SIR (to the right) on the first axis with no clear geographic pattern on the second axis (Figure 3c).

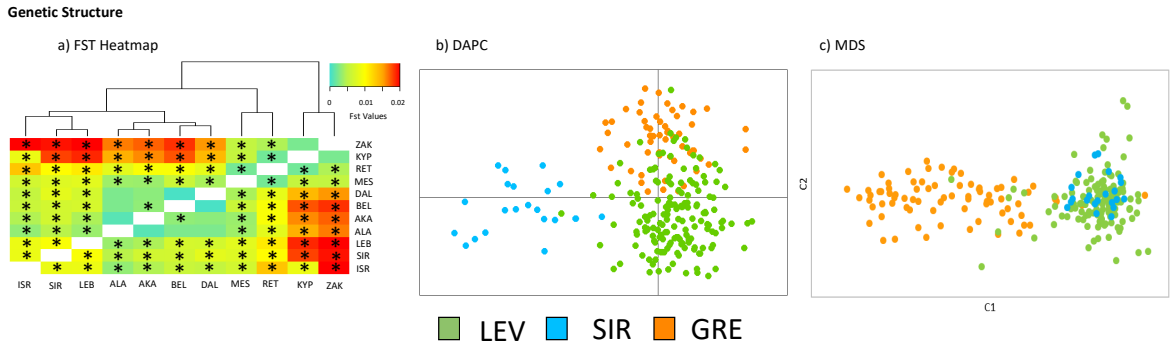

Figure 3: Genomic structure of the complete MED Baseline. Mediterranean nesting individuals (n=238) from 11 populations belonging to 3 SubRMUs previously described in Barbanti et al., -, in prep: LEV (Levantine; N=136), SIR (Sirte; N=22), and GRE (Greece; N=80) were analyzed and a total of 5,177 SNPs retained. a) Heatmap and dendrogram based on the FST pairwise population genetic distances (*, indicates the significance); b) Individual based Discriminant analysis of principal components (DAPC), and; c) Multi-Dimensional Scaling (MDS) analysis based on Identity by State (IBS) individual pairwise genetic distances. In b and c each dot represents an individual color-coded according to SubRMU.

Assessing individual assignment

The effectiveness of self-assignment accuracy and membership probabilities of each Baseline dataset was evaluated using the DAPC (Chambers & Hastie, 1992) and the 'assignPOP' v1.1.4 (Chen et al., 2018) R package. In the DAPC analysis we used the probabilities obtained with the 'predict' function to evaluate genetic differentiation. In the case of 'assignPOP' analysis, it was necessary to test all models provided by the package. For all Baseline data sets, the Support Vector Machine (SVM) model presented a better self-assignment accuracy (Figure S2), and consequently this model was selected for all the individual assignments. In the DAPC membership probability of the RMU Baseline and the RMU Baseline-R datasets, all samples were assigned with a full probability to the RMU in which they were sampled: NWA, NEA or MED (Table S4; Table S5). Additionally, in the 'assignPOP' self-assignment accuracy analysis with both

RMU Baseline, all samples were assigned with a high percentage of probability (>0.99) to their corresponding RMU: NWA, NEA or MED (Table S4; Table S5). In the analysis of the membership probability and self-assignment accuracy of the MED Baseline, two assignment approaches were designed, which correspond to the two different genetic cluster scenarios obtained in the genetic differentiation evaluation. (Figure 3b with three clusters obtained by DAPC and Figure 3c with two clusters obtained by MDS). The DAPC considering three genetic clusters (GRE, LEV and SIR) resulted in 209 individuals assigned to their genetic cluster of origin (Table S6) with a high membership probability (>0.99), and 29 individuals with the highest membership probabilities ranging from 0.87 to 0.40 thus being potential admixed individuals. However, the 'assignPOP' self-assignment accuracy analysis assigned all the individuals with a high probability (>0.99) to their origin genetic clusters (GRE, LEV or SIR). When considering only two genetic clusters (GRE and LEV+Sirte), we obtained 218 individuals (Table S7) assigned with DAPC to their origin with a high membership probability (>0.99) to their corresponding genetic clusters and 20 individuals with a membership probability ranging from 0.87 to 0.52, suggesting an admixed origin, while with 'assignPOP' all individuals were perfectly self-assigned (Table S7).

Individual assignments in foraging areas at RMU level

We evaluated the origin of the individuals sampled in the foraging grounds using individual assignment to their Regional Management Unit with 'assignPOP'. We used the two baseline datasets constructed to perform IA at this level, the RMU Baseline (Table S8) and the RMU Baseline-R (Table S9), thus considering NWA, NEA and MED as potential origins of the unknown individuals. Of the 103 individuals from foraging grounds 99 were assigned to the Mediterranean RMU with a high percentage of accuracy (>0.90), being probabilities higher with RMU Baseline. One individual from CAT and two from LAM were assigned to the NWA RMU, and one individual from LAM to the NEA RMU with both baselines (Figure S3, Figure 4). However, the 4 individuals assigned to the Atlantic RMU varied slightly in the accuracy percentages depending on the baseline dataset used, with the reduced dataset always yielding higher probabilities (Figure S3,

Figure 4) for the Baseline RMU-R dataset, especially for those individuals assigned to WAT.

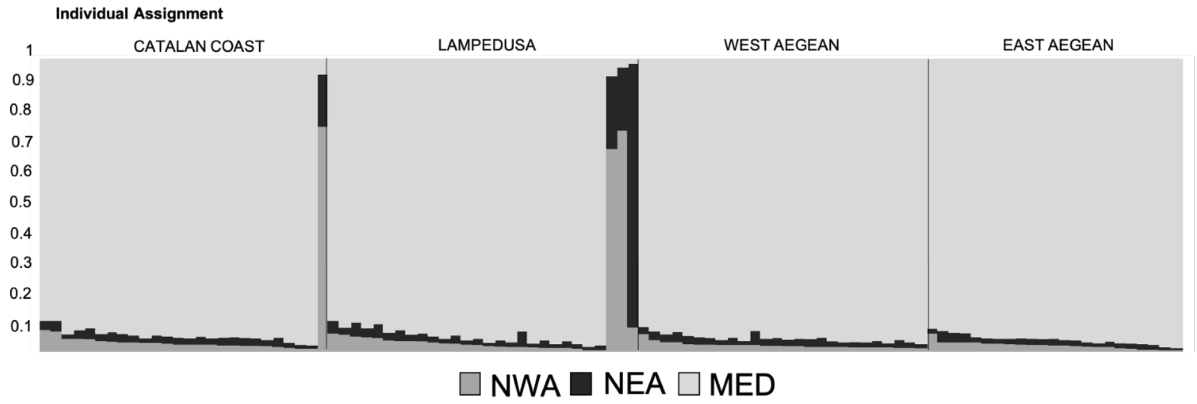

Figure 4: Assignment probabilities of the foraging ground individuals to each RMU using the RMU Baseline-R dataset. Each bar represents the assignation probability of one individual of the foraging grounds, as obtained with assignPop, to each one of the three putative RMUs of origin (NWA= North West Atlantic, NEA= North East Atlantic and MED= Mediterranean). The same loci as in RMU Baseline-R (4,321 SNPs) were kept for individual assignment of the 103 foraging ground individuals

Individual assignments in foraging areas at SubRMU level within the Mediterranean

Individuals assigned at the previous level to an Atlantic RMU were excluded, and only the 99 individuals found to be originated in the Mediterranean RMU were evaluated considering the two different genetic cluster scenarios in three and two SubRMUs obtained in the genetic differentiation analyses. We considered a successful assignment to a single SubRMU with a probability >0.7, while individuals with lower probabilities and distributed in different SubRMUs were considered potential admixed. In the analysis with three genetic clusters: SIR, LEV and GRE, from the 99 individuals from foraging grounds, we observed 23 individuals assigned to the region of Greece, 58 individuals assigned to the Levantine region and 5 individuals that were assigned to Sirte; 86 were assigned to a region with an accuracy >0.7, while the other 13 presented an admixed assignment to more than one region (Table S10). Although the individuals from the Levantine region were the most abundant, the origin of individuals varied among foraging grounds with Lampedusa having the largest number of individuals from Sirte and West Aegean with the larger number of individuals from Greece (Figure 5, Table 2).

Moreover, the Catalan Coast and Lampedusa presented the largest number of admixed individuals (Table 2). Most of the admixed individuals had high assignment probabilities to two SubRMUs, varying the combination in different foraging grounds (Figure 5). In the other hand, using the MED Baseline with two genetic clusters (GRE and LEV, which includes Sirte), we observed 95 individuals assigned to a single SubRMU , in addition to 4 individuals with an admixed assignment (Figure S5; Table S11). These 4 individuals were also included in the 13 individuals assigned as admixed in the analysis with 3 genetic clusters. The remaining 9 individuals had a high probability of being of LEV+SIR cluster and in the assignment of the three groups had intermediate probabilities of being of these two independent clusters (Figure 5). These individuals were distributed in all foraging grounds but mostly detected in the foraging grounds of the Catalan coast and Lampedusa (Table 2).

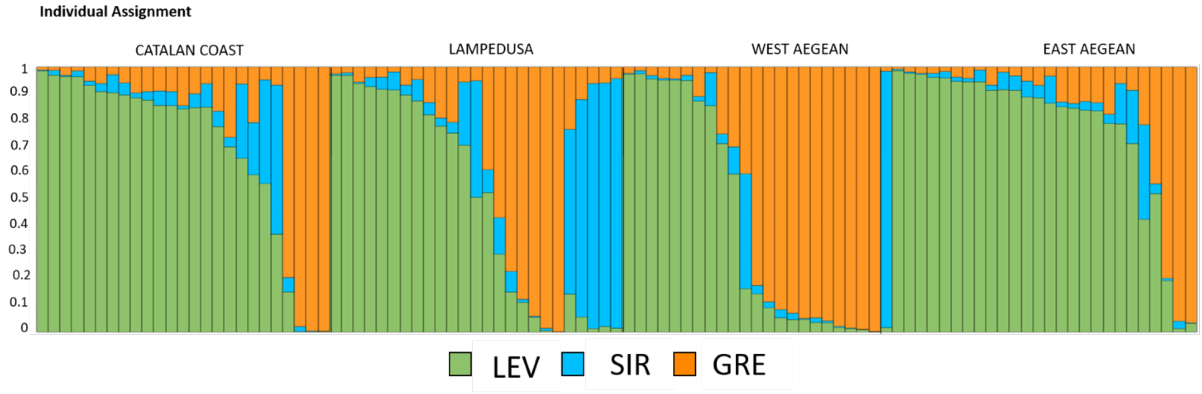

Figure 5: Assignment probabilities of the foraging ground individuals to each SubRMU using the MED Baseline and considering three genetic clusters. Each bar represents the assignation probability of one individual of the foraging grounds, as obtained with assignPop, to each one of the three putative RMUs of origin (LEV=Levantine Region, SIR=Sirte and GRE=Greek Region). The same loci as in MED Baseline-R (5,177 SNPs) were kept for individual assignment of 99 foraging ground individuals.

Table 2: Summary of the individual assignments at the Subregional Management Units level, of the individuals of Mediterranean origin in the foraging areas. Individual assignments of foraging ground individuals from Catalan Coast (CAT), Lampedusa (LAM), West Aegean Sea (WAS) and East Aegean Sea (EAS), considering 3 subregions (LEV=Levantine Region, SIR=Sirte and GRE=Greek Region) and 2 subregions (LEV+SIR=Levantine Region and Sirte, and GRE=Greek Region), in both analysis the admixed individual are presented as individuals that belong to more than one subregion (See Table S10 and Table S11 for details).

	Population Group	**Foraging Region**			
		CAT	**LAM**	**WAS**	**EAS**
MED Baseline 3 clusters	LEV	16	12	9	21
	SIR	0	4	1	0
	GRE	4	5	11	3
	ADMIXED	5	4	2	2
MED Baseline 2 clusters	LEV +SIR	21	18	11	22
	GRE	4	5	11	3
	ADMIXED	0	2	1	1

DISCUSSION

Non-model and highly migratory species often have complex life cycles and population structure, which can result in a challenge for population studies and for scientifically based management and conservation. In this study we used the latest genomic techniques on marine turtles to first describe the different levels of genetic structure in the loggerhead turtle Atlantic and Mediterranean nesting populations, and then use this genomic information as baseline to perform individual assignments on individuals found in foraging grounds in the Mediterranean. Our results provide novel insights into the biology of the species that are highly relevant for its management and conservation, but they also provide a methodology that can be replicated in other migratory species to link breeding populations with individuals in distant areas.

The hierarchical genetic structuring of the Atlanto-Mediterranean loggerhead sea turtles

The existence of intermediate levels of genetic structuring between the species and the population levels is rarely considered in population genetic studies. However, the existence of these intermediate levels can interfere with the analysis, thus demanding a hierarchical approach to unveil fine scale structuring (Carreras et al., 2020). In the case of marine turtles, the figure of the Regional Managements Units (Wallace et al., 2023; Wallace et al., 2010) was proposed to satisfy the need to have a conservation figure covering the common foraging areas used by a set of different genetic populations. However, although genetic data was used to identify genetically differentiated nesting populations, the support of the RMUs was obtained using other sources of data, such as satellite telemetry. For instance, a global population genetic study of the species using a fragment of the D-loop of the mtDNA revealed a deep genetic differentiation among nesting populations (Shamblin et al., 2014). However, the RMU structure was not apparent, with some populations within the same RMU being highly differentiated and some populations from different RMUs being not significantly different. This region of the mtDNA is characterised by presenting widespread highly frequent common haplotypes, and thus the power to detect genetic differentiation mostly relies on shifts in frequencies among

populations. The use of multilocus nuclear markers, such as microsatellites, allowed to assess the genetic structure at individual level and detected a deep genetic differentiation between the Mediterranean and the North West Atlantic RMUs, indicating that the populations in these two RMUs were likely to be isolated despite having individuals sharing common foraging areas (Carreras et al., 2011) although no samples were taken directly from the NWA RMU nesting populations. The present study uses a large set of SNPs on samples covering three different RMUs in the Atlanto-Mediterranean region and is the first study to demonstrate that these three RMUs can be fully supported only by genomic data, thus being a real intermediate level of genetic structuring in the species.

An additional interesting result is the degree of differentiation we found among the three RMUS, with the Mediterranean RMU being the most differentiated from the two Atlantic RMUs. The phylogeography of the species has some key unresolved aspects such as how the species expanded worldwide and colonised the different regions. One hypothesis suggested that the colonization of the Mediterranean was produced by individuals from the western Atlantic rookeries (Shamblin et al., 2014) while other authors suggested that this colonization was produced by individuals arriving from the Indian ocean, through the South African route, with a posterior migration of individuals to the already existing Atlantic RMUs (Baltazar-Soares et al. 2020). However, none of these studies included genome wide nuclear information and thus the hypothesis rely only on a mitochondrial marker which may reveal contrasting phylogeographic hypothesis in relation to the nuclear genome (Galià-Camps et al., 2024). Future studies using genomic data on an extended set of populations could be key to resolve these questions, as done with invasive species with complex evolutionary trajectories (Galià-Camps et al., 2022).

The Regional Management Units are not the only intermediate level of genetic structuring found for the species above the population level. The reanalysis of published genomic data (Barbanti et al., -, in prep) confirmed the existence of genetic structure at various

levels within the Mediterranean RMU. The first efforts to define the genetic structure in marine turtles were based on mtDNA and microsatellites and focused primarily to detect genetically differentiated populations in order to define Management Units for conservation (MUs, Moritz 1994). Initially four independent units were described in the Mediterranean (Carreras et al., 2007), a number that was increased to five (Clusa et al., 2018) and seven (Shamblin et al., 2017; Shamblin et al., 2014) depending on the sample sites but also on the type and number of markers used, as increasing the number of markers increased the ability to detect genetic differentiation Clusa et al., 2018. However, using 2bRAD data from previous studies (Barbanti et al., -, in prep) almost all populations resulted to be significantly different, with a few exceptions including localities of the Levantine region (Cyprus and Turkey) or mainland Greece, indicating a deep structuring driven by philopatry and highlighting the importance of the number of markers in population genetics. Besides a better resolution of structuring at population level, one of the most interesting results when analysing the samples from the Mediterranean RMU is the detection of genetic clusters grouping differentiated populations. Our genomic analysis with the Eastern Mediterranean nesting areas resulted in 3 main groups that could constitute another hierarchic level to be considered: 1) Sirte; 2) Levantine, composed by ISR, LEB, ALA, AKA, BEL, DAL; and 3) Greece, that included MES, RET, KYP, and ZAK, as already indicated in the previous study (Barbanti et al., -, in prep), although some analysis suggested only two. This grouping is not unprecedented, as it aligns with the predictions of hatchling dispersal from rookeries inferred from particle modeling (Casale and Mariani 2014). This study proposed the creation of an intermediate of conservation unit below the RMU to group populations with similar hatchling dispersal trajectories and proposed the name of subregional management units. With the confirmation of the SubRMUs as being also an intermediate level of genetic structure the hierarchical genetic structure of the loggerhead turtle in at least three levels (populations, SubRMUs and RMUs) is confirmed. This complexity needs to be considered when building a baseline to assess the origin of turtles at sea.

Individual assignments on marine turtles using genomics

Our testing of the potential of genomics for individual assignments revealed some technical questions that needed to be considered when using genomic data. The genotyping strategy was relevant, as genotyping all the unknown samples simultaneously with the baseline samples using the reference genome recovered all the polymorphic markers of the baseline. On the contrary, while performing the genotyping of the unknown individuals independently, we did not recover the markers that may be polymorphic in the baseline but not among the unknown samples. To optimize informatic resources and reduce the output file, BCFtools performs the genotyping by retaining only the positions of the raw data showing polymorphism in relation to the reference genome (Li 2011). This implies that the number of loci recovered is highly dependent on the number of samples genotyped at once and the genetic diversity they present. Consequently, an independent genotyping can cause dramatic loss of markers when the number of unknown individuals to assign is low, compromising its correct assignment. Thus, maintaining the variability of the samples provided here with a joint genotyping at the time of analysis could ensure a higher number of markers and sufficient genetic variability to increase the probability of assignment. For this reason, we considered the joint genotyping as the best strategy in future studies.

Another relevant issue was whether the number of samples across the different potential genetic clusters of origin of the baseline could bias the results of both the population genetic structure analyses and the individual assignments. In our case, this question was particularly relevant when analysing the data at RMU level, as the number of samples across the three RMUs was uneven with an overrepresentation of the Mediterranean RMU. Using all the information (RMU Baseline dataset) or using a reduced dataset (RMU Baseline-R dataset) had different effects depending on the analyses performed. While the FST and DAPC analyses showed almost no differences between the two datasets, the MDS with the complete dataset was less efficient at differentiating the two Atlantic RMUs. These results were not unexpected, as uneven sampling sizes are

known to interfere with some analyses such as MDS (Chapter 3.2). The difference in the clusters obtained by DAPC and MDS, despite using the same data set, can be explained to the differences in the objectives and approaches of these two statistical methods. On one hand, the DAPC maximizes the variance among groups and minimizes the variance within groups by using prior group information relying on known group membership information. On the other hand, the MDS seeks to represent the distance or similarity between individuals in a lower-dimensional space focused on maximizing the percentage of distance variability explained by the axes without imposing group restrictions. Thus, DAPC can reveal clear structures between predefined groups, identifying the axes that best separate the known groups while MDS rather reflects the overall variability in the data (Chambers & Hastie, 1992; Purcell et al., 2007).

Regarding the individual assignments, the first important observation in the analysis with both RMU baseline datasets is that all the individuals were consistently assigned to the same RMU in each test. This indicated that the genetic variability constituting the baseline is sufficient to differentiate at the Atlantic-Mediterranean level regardless of sample sizes. However, the individuals from the foraging grounds assigned to the Atlantic RMUs had lower probabilities when using the complete dataset. Thus, although both analyses were robust, reducing sample size differences across the potential origins in the baseline can be crucial to improve the individual assignments of unknown individuals of the underrepresented group. Additionally, in both analyses, individuals from foraging grounds were assigned to the MED and NEA RMUs with a higher probability than to the NWA RMU. These differences could be produced by the reduced representation of nesting populations in this RMU, which may not be robust enough to represent all the genetic variability of this RMU. Therefore, an opportunity exists to increase the sampling of the NWA RMU, to provide a better representation of its genetic variability in the baseline, consequently improving the assignment power. For example, in the southeastern United States, the Florida coast supports one of two largest loggerhead turtle (*Caretta caretta*) aggregations worldwide (Shamblin et al., 2011), while the Caribbean Sea, including

the Cuban Archipelago and Colombia's Caribbean coast, represents important nesting grounds none of them included in our dataset. Including greater genetic variation in the Atlantic Baseline representation would be beneficial to improve the resolution of the baseline.

In any case, the power of all baseline datasets was confirmed by the high self-assignment accuracy and membership probabilities analysis, demonstrated either by DAPC (Chambers & Hastie, 1992) or the 'assignPOP' v1.1.4 R package (Chen et al., 2018). Finally, the selection of the model is also a crucial step. With the SVM model consistently accurately describing the baseline according to Monte Carlo cross-validation in the assignment accuracy in all the tested scenarios. This model had better performance probably because the effectiveness in analysing high-variance datasets, maximizing the margin of separation between classes, and its robustness against outliers, allowing for the consideration of specific variability at the group level (Ripley et al., 2016).

Once these technical issues were solved, we were able to perform reliable individual assignments at the level of RMU of all samples and at the level of SubRMU of the individuals assigned to the Mediterranean RMU. The IA outperform MSA when assessing the origin of individuals in foraging areas as they can work at individual level and are not associated to large confidence intervals (Carreras et al., 2011). The approach used here represents a significant improvement in several aspects. The IA based on mtDNA and microsatellites considered only two RMUs, the NWA and the Mediterranean, as baseline samples from the NEA were not available (Carreras et al., 2011). Furthermore, the ratio of individuals not assigned to any RMU due to a lack of resolution or amplification problems was on average from 13% (Clusa et al., 2016) to 22% (Piovano et al., 2011) but reached 35% of failed assignations in some foraging areas (Clusa et al., 2016). On the contrary, our genomic approach was able to identify all the 103 samples from foraging individuals to a single RMU with high assignation probabilities and identified individuals from all the three RMUs of the baseline. Consequently, in this

study, we achieved a complete assignment to the RMU of origin, which we attribute to the increased number of markers used and the extended baseline. Furthermore, a hierarchical approach allowed for the first time to perform IAs to determine the origin from specific SubRMUs within the Mediterranean RMU. However, when we conducted the analysis considering three SubRMUs, 13 individuals exhibited admixed assignment and 4 while considering only two SubRMUs. We observed that the number of genetic clusters used in the assignPOP tool influences the assignment, as it tends to force the assignment to the genetic cluster that contains the locality most closely associated with the unknown individuals. Moreover, the admixed individuals when using the three SubRMUs, that are not identified as admixed with the two SubRMUs assignment, show in the former intermediate probabilities for Libya and the Levantine region, two groups that are combined in the latter. The presence of admixed individuals of these SubRMUs is not unprecedented, as they have also been reported in individuals nesting in these same populations (Barbanti et al., -, in prep) indicating that these intermediate assignations most likely correspond to true admixed individuals rather than being unassigned due to a lack of resolution of the methodology. In summary, this is the first study to report the identification of origin at a subregional level within the Mediterranean, as well as to document the admixture of origin with unprecedented precision for individuals found in foraging areas.

Heterogeneity of the foraging grounds in the Mediterranean

The application of the genomic-based IA indicated that individuals from different origins (NWA, NEA and MED) are found in aggregations during juvenile stages in the Mediterranean and that the composition of origins was very different among the different foraging areas. This is consistent with previous reports using mitochondrial MSA, which also suggested that this heterogeneity associated with foraging areas can be related to the prevailing currents in the area (Carreras et al., 2006; Clusa et al., 2014). The presence of Atlantic individuals along the Catalan Coast and in Lampedusa, but not in the Aegean Sea, aligns with the observed presence of Atlantic individuals in the western and central Mediterranean (Carreras et al., 2006; Clusa et al., 2014). However,

one of the most surprising results from our study is that the percentage of individuals assigned to an Atlantic RMU was significantly lower compared to percentages reported in previous studies, with up to 20-30% of the individuals in the Catalan Coast and 90% in Lampedusa (Carreras et al., 2006) determined to be from Atlantic origin. However, caution is warranted when making these comparisons, as the previously accepted confidence intervals were quite large and the methodology, both in terms of markers and analysis, were very different. However, these striking differences can also be indicative of potential differences in the composition of the mentioned foraging grounds over the years. This reduction in the percentage of Atlantic individuals can have different non-exclusive explanations. On one hand, the Mediterranean nesting populations have recently experienced a recovery as the result of the conservation efforts (Mazaris et al., 2017). Consequently, the arrival of more individuals of Mediterranean origin to the mentioned foraging grounds could have diluted the presence of Atlantic individuals.

Additionally, the reported alteration in the Atlantic Meridional Overturning Circulation (AMOC), a massive oceanic "conveyor belt" that redistributes heat via currents and acts as a nutrient distribution guide for species, could be influencing the proportion of Atlantic Sea turtles in the Mediterranean, as they encounter a non-physical geographical barrier limiting their arrival into de Mediterranean (Ditlevsen & Ditlevsen, 2023). In any case, future studies using the same methodologies in the same foraging areas but in different periods are needed to assess the potential shifts in the composition over the years with particular attention to future changes caused by global warming of population dynamics.

Even though almost all the individuals sampled in the foraging areas originated in the Mediterranean RMU, the fine scale resolution at the SubRMU level highlighted that the composition of individuals at different foraging grounds were different even at small scales. This study is the first to report IA at the SubRMU level, but some insights about the distribution of the individuals at this scale across the different foraging grounds were reported using mtDNA MSA (Clusa et al., 2014), although only the Catalan coast from our set of foraging grounds was analysed in this study. The authors suggested

that this distribution was driven primarily by the main currents in the Mediterranean. One of the major differences between the studies, besides the low number of detected Atlantic individuals, is the reduced number of individuals from Libya we found in the Catalan coast compared to this previous study with 39% of the individuals coming from this region. As mentioned before, the MSA is known to produce high interval confidence due to the presence of common haplotypes, in this particular case with a standard deviation of 29%. Although future studies are needed to check for potential temporal variations in the composition of foraging grounds, our results highlight the risks of relying on analysis such as the MSA considering only the mean values without their associated error. Besides previous knowledge, our results also align with the hypothesis of a migration from nesting areas to foraging grounds driven by the main current system (Millot & Taupier-Letage, 2004) and with the predictions of hatchling dispersal obtained using particle modelling (Casale & Mariani, 2014). In agreement with these studies, the foraging areas of the Aegean Sea were used by individuals from Greece and Levantine region. It is remarkable that both coasts of the Aegean Sea showed clearly different composition, with a higher contribution of Greece in the west coast (the Greece coast) and a higher contribution of individuals from the SubRMU of the Levantine in the east coast (the Turkish coast). Similarly, Lampedusa showed a mixed composition of individuals from the three SubRMUs, although with a relatively high proportion of individuals from the Levantine. Finally, the overdominance of individuals from the Levantine in the Catalan coast, with a reduced representation of individuals from Greece, is unprecedented. One possible explanation to these changes in relation to previous studies could be the recent expansion of the Turkish populations in relation to the remaining nesting populations of the Mediterranean (Casale et al., 2018), that could explain, not only the composition of the Catalan coast, but the general dominance of juvenile individuals from the Levantine region in the foraging areas. Future studies using the same methodology in other foraging areas are advised to provide a more complete picture of the migration routes of the juveniles in the Mediterranean.

How to perform future Individual Assignments in Marine turtles

In this work, we propose a methodology that can be widely used to study the genetic structure of individuals in foraging grounds across their Atlantic-Mediterranean distribution, and even serve as a guide for the design of genomic baselines for other highly migratory species. We provide a genomic baseline for assigning individuals of unknown origin at different levels, as well as the list of markers used. The following steps are established for this process (Fig. 6): 1) use 2bRAD with base selective-adaptors, Chapter 3.2) to sequence your samples; 2) genotype your new samples jointly with the RMU baseline provided in this paper; 3) use the –keep filter in VCFtools (Danecek 2011) to generate a file containing the unknown individuals; 4) retain in this VCF file the same SNPs as in the selected baseline dataset using the –snps filter (Danecek 2011); 5) generate a GENEPOP file for each VCF file; 6) use assignPOP to determine the origin of the individuals, considering the populations used in this study. Note that the number of retained markers may vary depending on the baseline used. We have provided the list of markers for each baseline dataset (SNPs list: RMU Baseline in File S1, RMU Baseline-R in File S2 and MED Baseline in File S3).

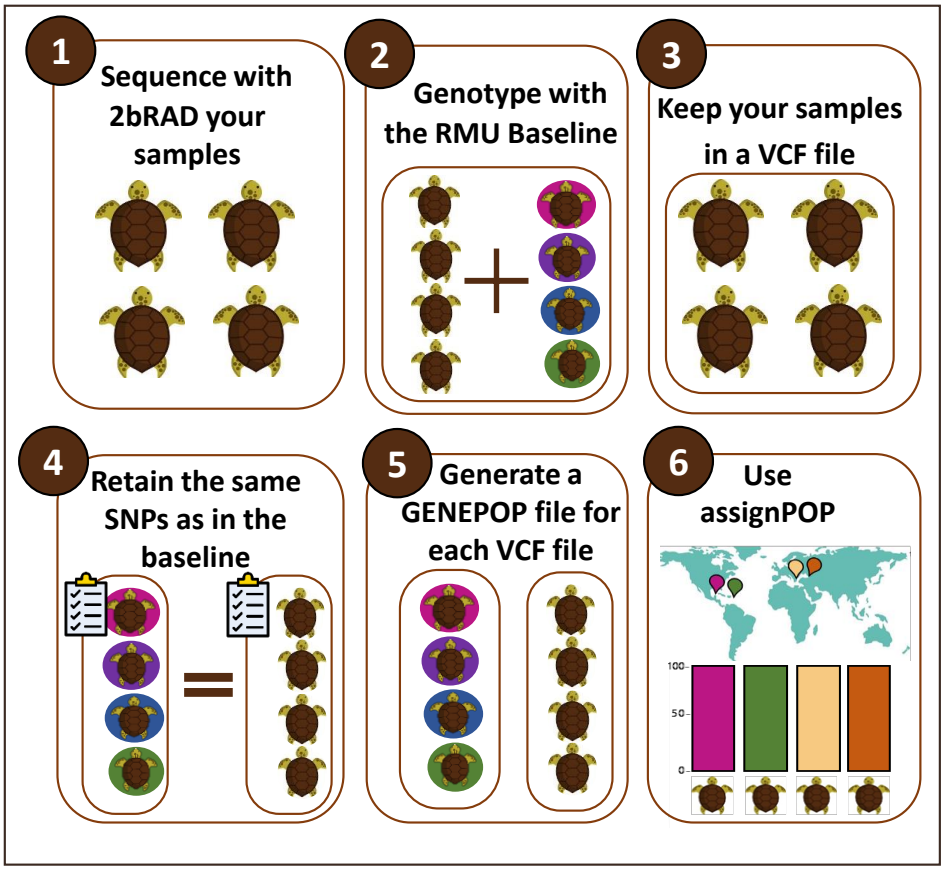

Figure 6: Flowchart of the proposed steps to assign individuals of unknown origin. 1) use 2bRAD to sequence your samples; 2) genotype your new samples jointly with the provided RMU baseline (present work); 3) use the −−keep filter in VCFtools to generate a file containing the unknown individuals; 4) retain in this VCF file the same SNPs as in the baseline using the −−snps filter; 5) generate a GENEPOP file for each VCF file; 6) use assignPOP to determine the origin of the individuals, considering the populations used in this study.

Our results demonstrate the feasibility to assess complex hierarchical structuring and to perform successful individual assignments in highly migratory species by using high-resolution genome-wide markers. Finally, we propose a methodology for future Individual Assignments of *C. caretta* individuals in the foraging grounds of the Atlanto-Mediterranean region, which can also serve as a guide for designing baselines for individual assignment in other non-model, highly migratory species, key to provide relevant information in species with complex life cycles for its management.

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SUPPLEMENTARY MATERIAL

SUPPLEMENTARY MATERIAL

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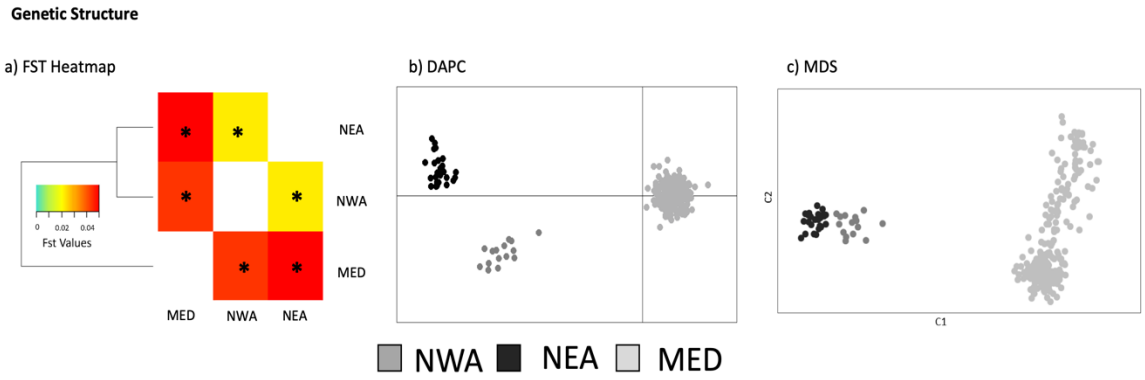


Figure S1. Genomic structure of the complete baseline to perform individual assignments at RMU level. The complete baseline (RMU Baseline dataset) included all analysed nesting individuals of Caretta caretta (n=278) from 3 RMUs (NWA=North West Atlantic, NEA=North East Atlantic and MED=Mediterranean), and a total of 5,308 SNPs retained. a) Heatmap and dendrogram based on the FST pairwise genetic distances between RMUs (*Indicates significant differentiation); b) Individual based Discriminant analysis of principal components (DAPC), and c) Multi-Dimensional Scaling (MDS) analysis based on Identity by State (IBS) individual pairwise genetic distances. In b and c each dot represents an individual color-coded according to RMU.

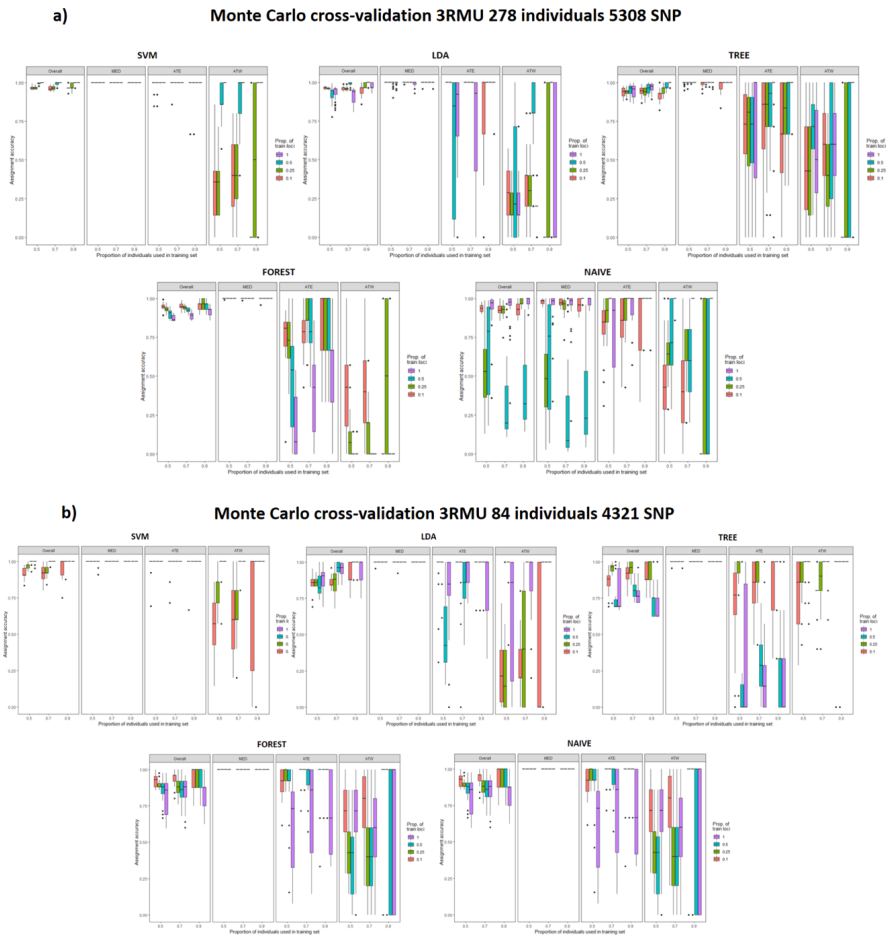
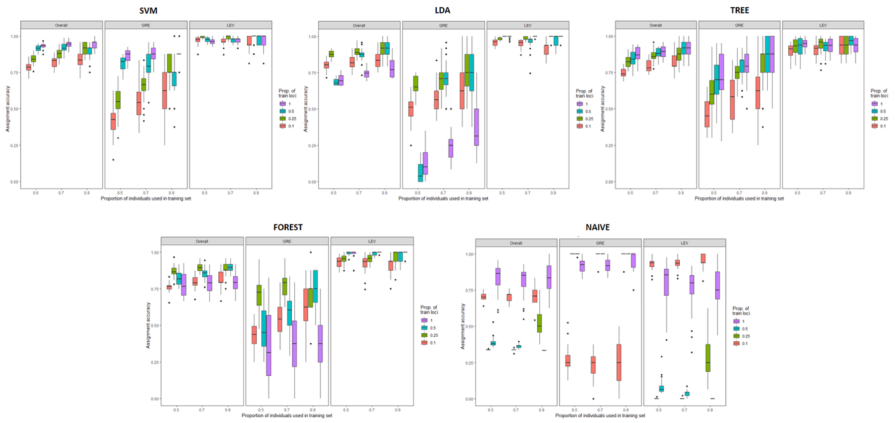


Figure S2. Model comparison for assignment accuracy in population classification. Assignment accuracy values are shown for each tested model: Support Vector Machine (SVM), Linear Discriminant Analysis (LDA), Decision Trees (TREE), Random Forests (FOREST), and Naive Bayes (NAIVE). The models were evaluated using all combinations of training loci proportions and training set sizes, applying the Monte-Carlo cross-validation method. The results demonstrate the variation in each model's ability to correctly assign individuals to their source populations based on the reference dataset. a) Monte Carlo cross-validation for the complete RMU Baseline with 5,308 SNPs, b) Monte Carlo cross-validation for the reduce RMU Baseline-R with 4,321 SNPs, c) and d) Monte Carlo cross-validation for the MED Baseline with 5,177 SNPs considering 2 and 3 genetic clusters respectively.

c) Monte Carlo cross-validation Mediterranean Region 238 individuals 2 Populations 5177 SNP



d) Monte Carlo cross-validation Mediterranean Region 238 individuals 3 Populations 5177 SNP

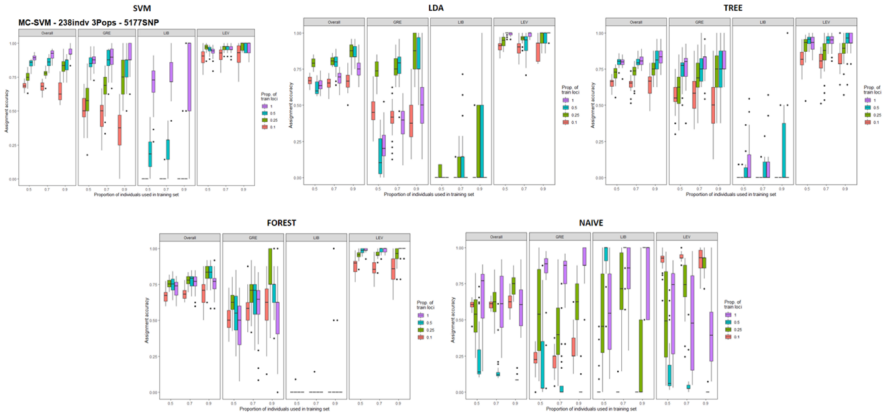


Figure S2. [Continuation] Model comparison for assignment accuracy in population classification. Assignment accuracy values are shown for each tested model: Support Vector Machine (SVM), Linear Discriminant Analysis (LDA), Decision Trees (TREE), Random Forests (FOREST), and Naive Bayes (NAIVE). The models were evaluated using all combinations of training loci proportions and training set sizes, applying the Monte-Carlo cross-validation method. The results demonstrate the variation in each model's ability to correctly assign individuals to their source populations based on the reference dataset. a) Monte Carlo cross-validation for the complete RMU Baseline with 5,308 SNPs, b) Monte Carlo cross-validation for the reduce RMU Baseline-R with 4,321 SNPs, c) and d) Monte Carlo cross-validation for the MED Baseline with 5,177 SNPs considering 2 and 3 genetic clusters respectively.

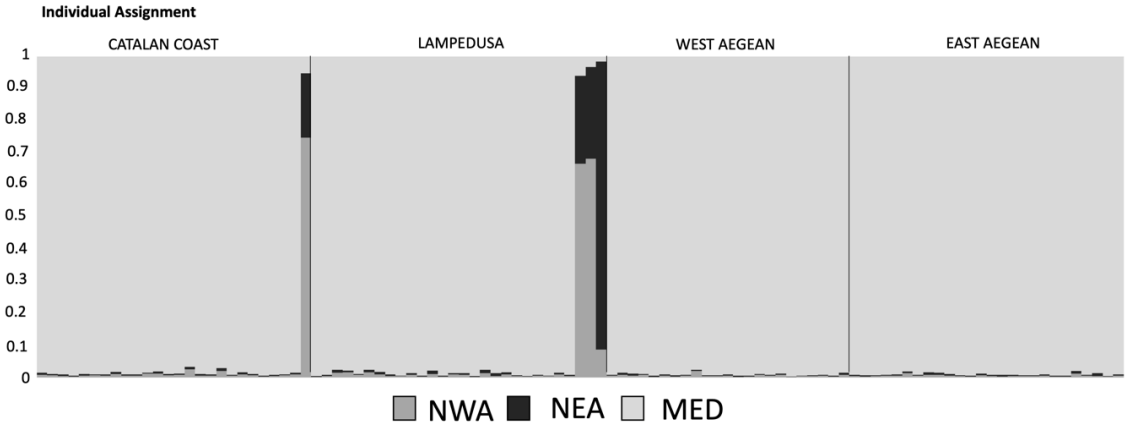


Figure S3. Assignment probabilities of the foraging ground individuals to each RMU using the RMU Baseline dataset. Each bar represents the assignation probability of one individual of the foraging grounds, as obtained with assignPop, to each one of the three putative RMUs of origin (NWA=North West Atlantic, NEA=North East Atlantic and MED= Mediterranean) The same loci as in RMU Baseline (5,308 SNPs) were kept for individual assignment of the 103 foraging ground individuals.

CHAPTER 3.2

NEW COLONISERS DRIVE THE INCREASE OF THE EMERGING LOGGERHEAD TURTLE NESTING IN WESTERN MEDITERRANEAN

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New colonisers drive the increase of the emerging loggerhead turtle nesting in Western Mediterranean

Astrid Luna-Ortiz[1,8], Gisela Marín-Capuz[1,8], Elena Abella[2], José Luis Crespo-Picazo[3], Fernando Escribano[4], Guillem Félix[5], Silvia Giralt[6], Jesús Tomás[7], Cinta Pegueroles[1,9], Marta Pascual[1,9] & Carlos Carreras[1,9]✉

The loggerhead sea turtle (*Caretta caretta*) is sensitive to climate change and is responding by colonising the Western Mediterranean. To understand the rapid nesting increase in recent years in Spain, we sampled 45 hatchlings from 8 nests between 2016 and 2019. We sequenced a mtDNA D-loop region, genotyped 2291 SNPs using 2bRAD and collected data on clutch size, hatching success, and incubation duration. We confirmed that the colonisation has a Mediterranean and Atlantic mixed origin and we detected that these nests were laid by different females, except for two nests within the same season. Our results suggest that the recent increase in nesting is due to an increase in the number of colonising individuals rather than females born in the same area returning to breed. We hypothesize that this increase in the number of colonisers results from successful conservation efforts, feminisation of the populations of origin and earlier sexual maturation. However, the percentage of offspring females produced in Spain suggests that future returning individuals will aid to the settlement of the new population. These results allow defining the current status of this colonisation although future efforts are needed to detect remigrants to confirm the establishment of a resident population.

Climate change is a major threat to global biodiversity[1]. The multiple components of this phenomenon are affecting directly all pillars of biodiversity and the species affected may respond in three ways: adapt, move, or become extinct. Some species are responding by shifting their geographic distribution or changing their phenology, altering their development and time of reproduction, modifying the composition of communities and their interactions[2,3].Consequently, extinction can be avoided if populations move to favourable habitats, organisms successfully overcome stressful conditions via plastic changes, or populations undergo evolutionary adaptation[4,5].

Among all marine species, sea turtles have a potential vulnerability to climate change, as multiple processes associated to this global phenomenon (e.g., increase of temperature, sea-level rise and increase of extreme meteorological events) can simultaneously affect these species during different stages of their lives and at large geographic scales[6,7].The increase in temperature is thought to cause a great impact on sea turtles because they have life history traits strongly influenced by environmental temperature[8,9]. For instance, sand temperature during egg incubation plays a vital role in embryo development, hatching success, hatching sex ratio due to their temperature sex determination and post-hatchling fitness characteristic[10–12]. Consequently, increases in temperature may skew population sex ratios towards females[13] collapsing the populations and compromising its long-term viability[14–16]. In addition, nesting beaches, especially reef islands, are likely to be impacted because of ocean acidification, affecting carbonate sediment production, sediment budget and sediment traits[17]. This

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potential alteration of the sediments is expected to affect sea turtles' reproduction, as they require specific sediment characteristics to incubate their eggs and dig their nests[18]. Potential impacts range from changes in hatchling emergence success to loss of nesting habitat suitability[19]. For instance, the size of grains in the sand plays a crucial role in both gas exchange and the facility for hatchlings to emerge from their nests[18,20]. In addition, changes in grain size and sorting can affect sand temperature[21], probably affecting sex determination. Considering all these potential impacts together, global warming is considered a major threat that jeopardises the viability of current nesting population[6,22].

Under this scenario, the use of new nesting sites and the colonisation of new areas can be crucial for the survival of sea turtle species. Humans can favour this process with actions such as breeding individuals in captivity, reintroducing individuals in natural environments or restoring altered nesting areas. Some management actions have promoted the successful recovery of sea turtle nesting wild populations or the establishment of new populations through the release of individuals in new areas to overcome the putative constraints to colonise new areas imposed by philopatry[23,24]. However, the study and management of natural colonisations should be considered a priority for the species survival before using assisted colonisations[25]. In this sense, Carreras et al.[26] analysed the sporadic nesting of the loggerhead turtle (*Caretta caretta*) in the Western Mediterranean and they discovered that these nesting events were due to colonisers from distant nesting areas in the western Atlantic and eastern Mediterranean and suggested that this natural colonisation was probably related to climate change. The authors predicted, using population modelling approaches, that this colonisation would raise rapidly under a global warming scenario. The increase of overall temperatures would favour the production of females in these new nesting events that would return to the new location when mature, in the process of becoming residents of the new population and reproduce in subsequent nesting seasons as remigrant. This colonisation would be later promoted by the philopatry of the species, as it implies that, once a sporadic nest is laid, the females born in this new nest would return to reproduce when they reach sexual maturity and might be detected in different nesting seasons (remigrant females)[27]. Consequently, detecting remigrant females nesting over subsequent nesting seasons would confirm the successful consolidation of a new population.

The predictions about a future increase of the nesting activity[26] seemed to be accomplished as an unprecedented number of nests started to be reported in the Western Mediterranean in the last decade[28], including the Spanish coast (Fig. 1a, b). This increase has implied that the nesting range of the species in the Mediterranean has been moving westwards associated to anthropogenic variables and sea surface temperature[29,30]. Two non-exclusive hypotheses can explain the increment of nesting activity in the western basin (Fig. 2). The first possibility is that the incipient population started to grow as the result of remigrant females that were born in the new location in the past that, after maturation, are currently returning to reproduce in the new area due to philopatry, as already detected in Conigli beach, in Lampedusa, Italy[26]. The second possibility is that the number of nests may be increasing because more colonising females are arriving at the new sites from their populations of origin. This increase in the number of arriving colonisers could be due to an increase in the number of females on the populations of origin, which might be a result of conservation efforts[31], due to feminisation of the populations[13,32], or due to both processes together. Another reason of the increase of the number of colonising nesting females could be related to an earlier maturation of the females in foraging areas related to an increase of sea temperature[29,33,34].

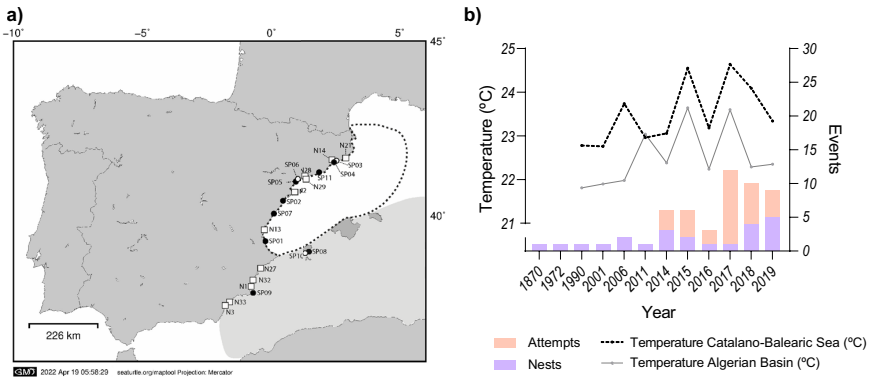

Figure 1. Summary of nesting activity in the Spanish coast. (**a**) Location of sporadic nests recorded in Spain from 1870 to 2019 (N = 22) as coded in Table 1 and Supplementary Table S1. Squares indicate nests laid between 1870 and 2015 in Spain as analysed in a previous study (N = 11)[26]. Circles indicate nesting events from 2016 to 2019 (N = 11) analysed in the present study, being the black circles, the nests analysed with 2bRAD sequencing (N = 8). Map created using MAPTOOL (SEATURTLE. ORG Maptool. 2002. SEATURTLE.ORG, Inc. http://www.seaturtle.org/maptool/ 18 Feb 2022)[97]. The two foraging areas in the region are highlighted with a black dashed line (Catalano-Balearic Sea) and a grey area (Algerian Basin). (**b**) Number of loggerhead turtle nests per year (N = 22) and attempts (N = 32) in the Spanish coast (Supplementary Table S1, Supplementary Table S2, adapted from Hochscheid et al.[28]. The lines represent the mean SST in June and July per year in the two foraging areas indicated in the map.

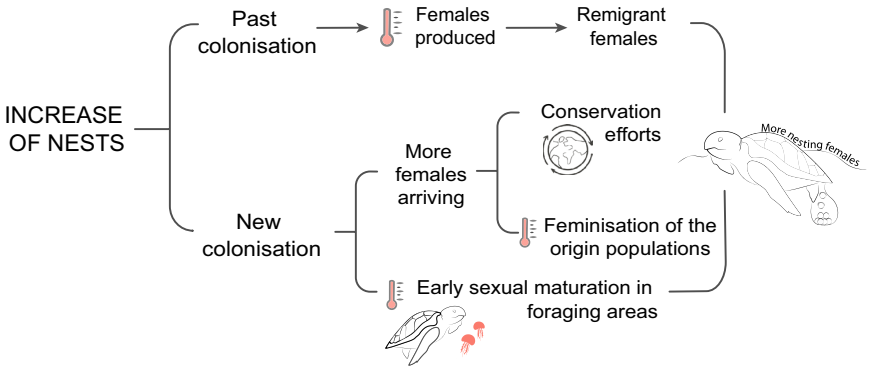

Figure 2. Non-exclusive hypotheses that may explain the increase in nesting activity in the Western Mediterranean basin. On one hand, females produced in past colonisation events could have returned upon maturation and established in the new areas as remigrant females. On the other hand, the increase of the nests could be related to an increase in the arrival of new colonisers. This increase could be produced by the arrival of new females, either due to the increase of the populations due to conservation efforts or due to the feminisation of the origin populations, of because of an early sexual maturation in foraging areas, increasing the chances of laying eggs outside of their origin nesting populations.

Understanding the emergence of new nesting areas has a strong evolutionary and conservation importance as it provides the opportunity to study colonisation-in-action events of long living philopatric animals[26]. Tagging-recapture studies in marine turtles, using either physical[35] or genetic tagging[36], have shown that the degree of philopatry is variable among and within species, and that deviations of tens of kilometres between nesting activities may happen. However, the presence of these non-strictly philopatric individuals could only explain short distance migrations along continuous nesting habitats, due to the short dispersion range. Therefore, sea turtles should have a mechanism for long-distance colonisation, as indicated by the widespread distribution of the species around the world[37], resulting from millions of years of evolution in environmental changing conditions. Likewise, the study of long-distance colonisation processes, such the one that is happening in the Western Mediterranean[26], is key to understand the origin and significance of both new and historical nesting events in sea turtles and to establish conservation plans in the context of the current global warming.

Research on marine turtles is challenging but studying an ongoing colonisation of marine turtles poses additional limitations. Scientific methods commonly used in regular nesting areas cannot be directly applied in this case due to the scattered distribution of nests along large coastlines[28]. In this context, genetic tools have been employed to gather reliable biological information from a limited number of samples when nesting is scarce, particularly in cases when detecting nesting females proves challenging[36]. However, genetic markers such as mitochondrial genes are limited when assigning nests to specific females or inferring the adult breeding population[26]. Nuclear markers are useful for this purpose, but the number of markers is a key factor in the analysis of genetic differentiation[38]. Thus, genomic methods are preferred when studying newly colonised marine turtle nesting sites. Due to the thousands of loci recovered with these techniques, thorough analysis is possible with only a few samples, providing essential information for conservation purposes[39].

In the present study we aim to understand the phenomenon of the long-distance dispersal and colonisation to new suitable shores by loggerhead turtles, testing the hypotheses that have been suggested (Fig. 2) to explain the recent increase in the nesting events in the Western Mediterranean. To do so, we explored the genetic composition of sporadic nests laid on the Mediterranean coast of Spain from 2016 to 2019 and we combined this genetic data with reproductive and environmental information collected in the nesting locations. Besides the inherent scientific interest, this work will be of importance when designing new conservationism protocols to aid the establishment of the loggerhead turtle on Spanish coasts.

Results

In Spain's Mediterranean coasts, the number of loggerhead turtle nesting events have been increasing since 2001[40] and since 2014 the species is nesting annually showing an increasing trend[28] (Fig. 1b). We gathered information on the 11 nesting events recorded over 2016–2019 years (Table 1, Supplementary Table S1), obtaining detailed data on all of them except from nests SP02, SP06, and SP11 as only hatchlings on the beach were found and clutches were not found. The mean clutch size per nest was 97 eggs (SD ± 35.11) and all nests but one (SP10) yielded viable hatchlings with hatching success ranging between 0% and 93.1%, with a mean of 55.56% (SD ± 29.12), showing also a high variability across nests (Supplementary Table S1). All clutches were laid in the summer season, between the months of July and August and hatched between the months of August and October (Supplementary Table S1). Rates of female offspring obtained from incubation durations ranged from 0 to 100%, with some variability within nest depending on the models used (Supplementary Table S1). We found a female-biased sex ratio in all but two of the nests analysed in this study (SP01 and SP07) that showed a male biased sex ratio. The habitat suitability of the nesting locations, according to estimated published map models

ID	Year	*Ld*	*Ed*	Beach (Locality)	Samples analysed	mtDNA		Polymorphic nuclear loci (%)	Mean Ho ± SD	Mean relatedness
						Haplotype	Haplotype origin			
SP01	2016	03/07/2016	05/09/2016	Les Palmeres (Sueca)	2	CC-A2.1	Shared	43.39	0.255 ± 0.01	0.082
SP02	2017	N/A	11/10/2017	Migjorn (Peñiscola)	2	CC-A1.1	Atlantic	38.19	0.253 ± 0.006	0.180
SP03	2018	15/06/2018	08/08/2018	Sant Simó (Mataró)	–	–	–	–	–	–
SP04	2018	01/08/2018	28/09/2018	La Descàrrega (Premià de Mar)	8	CC-A3.1	Shared	48.8	0.253 ± 0.008	0.235
SP05	2018	N/A	16/09/2018	Vilafortuny (Cambrils)	4	CC-A2.1	Shared	43.3	0.275 ± 0.035	0.301
SP06	2018	N/A	24/09/2018	Ardiaca (Cambrils)	–	–	–	–	–	–
SP07	2019	13/07/2019	14/09/2019	Del Serradal (Castellón de la Plana)	7	CC-A31.1	Mediterranean	49.24	0.279 ± 0.046	0.229
SP08	2019	25/07/2019	10/09/2019	D'en Bossa (Sant Jordi de ses Salines)	7	CC-A2.1	Shared	52.68	0.317 ± 0.051	0.221
SP09	2019	28/07/2019	18/09/2019	Calblanque (Cartagena)	7	CC-A2.1	Shared	50.72	0.283 ± 0.045	0.216
SP10	2019	29/07/2019	N/A	D'es Cavallet (Sant Francesc de s'Estany)	–	–	–	–	–	–
SP11	2019	N/A	06/10/2019	Castelldefels (Castelldefels)	8	CC-A31.1	Mediterranean	47.4	0.243 ± 0.006	0.225

Table 1. Sporadic nests of loggerhead turtle in the Spanish coast from 2016 to 2019 and genomic data associated. *Ld*, Date of egg laying; *Ed,* date of first emergence; mtDNA, nest haplotypes and attributed nesting area to each haplotype; Polymorphic loci, percentage of polymorphic nuclear markers per nest; *Ho*, observed heterozygosity; Mean relatedness, relatedness per nest inferred using Manichaikul relatedness index[44]. N/A indicates data is not available. A value of (–) indicates that no genetic data could be obtained from these nests. Additional information of these nests can be found in Supplementary Table S1.

for the Mediterranean region based on nine independent terrestrial temperature and precipitation variables[41], categorized the locations as "Marginal", "Good" and "Excellent"[41], and with a generally predicted to increase in hatching success in the future[42] (Supplementary Table S1).

SST in surrounding foraging areas during nesting period

All events that occurred between 1990 and 2019 in the Spanish coast (including attempts and nests, Supplementary Table 1–2) were in areas with a sea surface temperature (SST) above 21 °C (Fig. 3), which is considered the temperature when the nesting season starts[34]. We detected a significant correlation between the annual number of total events in the Spanish coast (Supplementary Table 1–2) and the mean SST in the same nesting period in the Catalano-Balearic region (Spearman' rank correlation, $\rho = 0.825$; p-value = 0.003), but not the Algerian Basin mean SST (Spearman correlation, $\rho = 0.554$; p-value = 0.096).

Genetic analyses

We obtained genetic data from 45 samples from 8 of the 11 nesting events that occurred from 2016 to 2019 nesting seasons. DNA extractions from samples of nest SP03 failed due to bad preservation conditions, no development was observed for any of the eggs of nest SP10 (Supplementary Table S1) and no samples were collected from nest SP06 by the authorities that attended these nesting events, although free hatchlings were observed on the beach and no clutch was found.

We obtained a total of 4 different D-Loop mtDNA haplotypes in the 8 nests (Table 1), all of them previously found in other areas[43]. The samples from 5 nests had haplotypes that can be found both in the Atlantic and in the Mediterranean nesting beaches (CC-A2.1: SP01, SP05, SP08, SP09; CC-A3.1: SP04). One nest had a haplotype that is exclusive to the Atlantic nesting populations (CC-A1.1: SP02), while the remaining two nests shared a rare haplotype that has been only found in Mediterranean nesting beaches (CC-A31.1: SP07 and SP11).

With 2bRAD sequencing we obtained a total of 246,481,025 raw reads (Supplementary Table S3), with an average and standard deviation of 5,477,356.11 ± 1,583,759.95 reads per individual. The percentage of mapping to the reference loggerhead genome was on average (SD) 93.8% (6.3) (Table S3). We detected a total of 154,613 non-filtered SNPs across all samples. After applying all filters and selecting only loci shared by at least 95% of the individuals, we retained a total of 2,291 loci, with an average depth of 25.07 reads per locus.

The percentage of polymorphic loci in each nest was variable (mean 46.72% of the loci; SD ± 4.75, Table 1). The observed heterozygosity value across all samples was similar (mean Ho = 0.271 ± SD 0.04, Supplementary Table S3), and the same was true for the observed mean heterozygosity per nest (mean Ho = 0.270 ± SD 0.02, Table 1). The relatedness[44] values between individuals of the same nest were variable across nests (ranging from

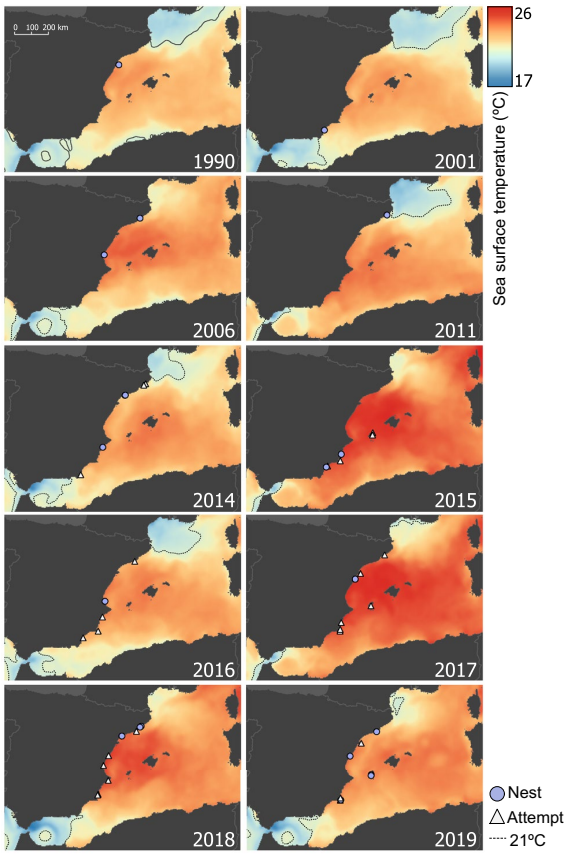

Figure 3. Spatial distribution of events detected by year (1990–2019) and the corresponding SST mean temperatures between June and July for each year. Only the SST gradient of the years with presence of nesting events (light blue circles) or attempts (white triangles) is illustrated. The isotherm of 21 °C SST, which is the threshold suggested as critical for the presence of nesting events in the Western Mediterranean[34] is represented with a dashed black line. Maps created using QGIS vs 3.22.9 software (https://www.qgis.org/en/site/).

0.082 to 0.3; mean 0.211; SD ± 0.062, Table 1), but generally much higher than between individuals from different nests (Fig. 4). The comparison of genotypes allowed us to identify re-nester females, defined as females laying multiple clutches, that could also be considered remigrant females if these clutches were laid in different years. Only in one case, the individuals from two different nests clustered together with relatedness values (nests SP07 and nest SP11), thus suggesting that the same female laid both nests (Fig. 4). Moreover, the pair of individuals from the nest SP01 presents lower relatedness values than other nests, suggesting possible multiple paternity within this nest (Fig. 4) as found in previous studies in the region[26]. Additionally, the MDS plot based on IBS distances generally clustered all the individuals from the same nest when considering the first three-axis while separated individuals from different nests (Fig. 5a). All individuals from the nests SP07 and SP11 clustered together with all three axes. However, three of the nests (SP01; SP02; SP05) also clustered in the centre of the three axes despite their low relatedness values (Fig. 4) and the different potential origin of the D-loop haplotypes, exclusive of the Atlantic for SP02 and shared among basins for SP01 and SP05 (Table 1). When only one randomly picked individual per nest was used to build the MDS plot, in order to avoid artificial clustering related to uneven sampling size, no clustering was observed except samples from the nests SP07 and nest SP11 (Fig. 5a). Interestingly, SP02 with a characteristic Atlantic D-loop haplotype was clearly separated by the first coordinate (Fig. 5b).

Discussion

Sea turtles are currently facing several climate warming impacts, ranging from rising of sea level to the thermal increase of ocean water and incubation conditions[45] which are predicted to be especially severe in the Mediterranean Sea[46,47]. As a probable adaptive response to the increased temperature, the loggerhead sea turtle is colonising the Western Mediterranean[26,29,48,49]. The nesting activity on the beaches in the Western Mediterranean has experienced an explosive increase in the last decade[28] with nesting becoming regular in some regions of Italy[48,49] suggesting that this colonisation process has started to consolidate[28]. By using genetic tools and in situ

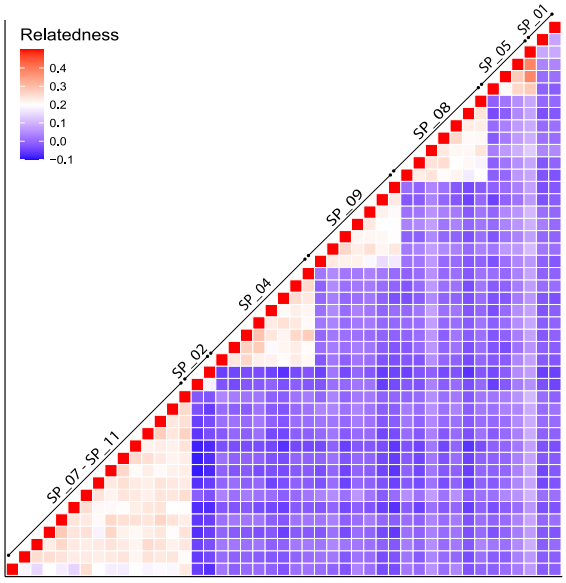

Figure 4. Heatmap based on the relatedness index among pairs of individual samples based on the 2,291 filtered SNPs obtained with 2bRAD sequencing. Each cell represents the value of the Manichaikul relatedness index[44] between sample pairs. The samples that belong to each nest are indicated in the diagonal arrows. Individuals from nests SP_07 and SP_11 clustered together. Nests are coded as in Table 1.

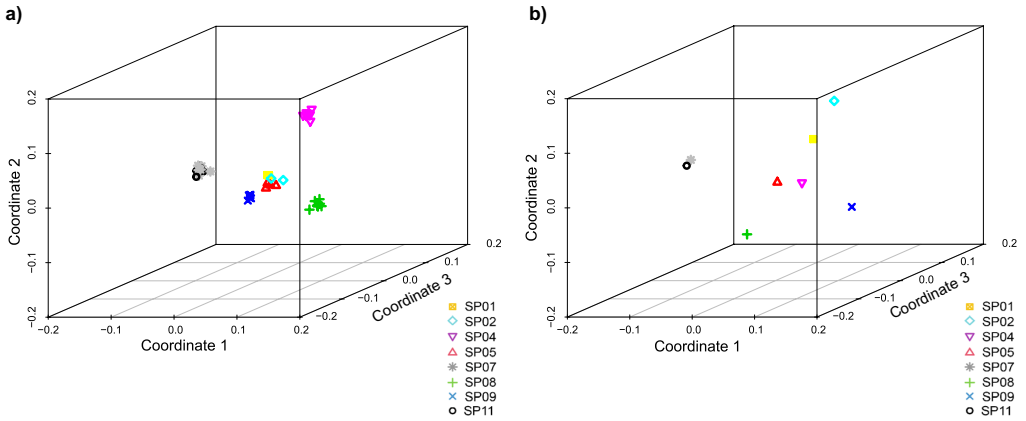

Figure 5. Multidimensional scaling (MDS) analysis plots based on IBS distance among the 45 individuals included in this study (2,291 SNPs), representing the genetic differentiation of the 8 nests analysed with 2bRAD sequencing on the Spanish coast. (**a**) 3D scatter plot considering all the individuals (N = 45). (**b**) 3D scatter plot, considering one individual per nest (N = 8). Nests are coded as in Table 1.

data collection, our results show that this emerging colonisation in Spain increased in numbers by non-related females laying eggs in different years, rather than because of the presence of remigrant females. However, considering the abundance of females produced because of the incubation conditions in the nests, there is potential to find remigrating females in the near future indicating the settlement of the new population.

In the present work, viable hatchlings were produced in most of the nests although with a high variability among nests (from 0 to 93.1% of hatching success), as reported in previous years in the Western Mediterranean region[26,28]. The reasons of this variability may be caused by nonexclusive factors, including local environmental conditions, different management strategies, or biological factors (body conditions of the female or genetic factors of both parents). Future research in emerging nesting sites is needed to unveil the reasons for these differences. A recent study analysing past temperature records has shown that the annual window for viable nesting in the Western Mediterranean (defined as the temporal window in which sand temperatures are suitable to host a viable nest during all its incubation) was small or absent until recently due to the cold-water temperature

but has increased over the past ten years[34]. If temperatures continue rising in the Mediterranean[1], the habitat suitability for loggerhead nesting will increase in the Western Mediterranean[45], but also the nests laid in these areas will produce a higher proportion of females[26,33]. Our results regarding the inferred sex ratio are in line with what has been found in other areas of the central and western Mediterranean[28,50], with a combination of nests with a high proportion of females but also some nests with a male biased sex ratio. Hence, given the natural philopatry of the species[33], the high percentage of female offspring could result in a potential increase of individuals returning as breeding adults, consolidating the establishment of the new population under suitable environmental conditions[26].

Our results also confirmed that the colonising individuals come from distant regular nesting areas in the Western Atlantic and eastern Mediterranean Regional Management Units (RMU)[37], as found in older nests[26]. The sequencing of the mtDNA D-Loop region revealed three nests that presented a haplotype exclusive from an RMU. Two of these nests (SP07-SP11) presented a haplotype (CC-A31.1) previously found in three non-related nests in Calabria and Kyparissia, regular nesting populations from the eastern Mediterranean[51], and in a nest from the Ionian coast of Sicily in 2010[52]. Additionally, the nest (SP02) presented a haplotype (CC-A1.1) that is exclusive from the Atlantic and widespread in almost all Western Atlantic populations[43]. The remaining five nests with genetic data presented haplotypes with a shared origin between both the Western Atlantic and Mediterranean regular nesting areas. These results align with previous studies[26], confirming a mixed presence of colonisers from different populations in the Western Mediterranean, and is consistent with the presence of juveniles and subadults from both regular nesting regions in nearby foraging areas[26,53]. Considering that the Atlantic and Mediterranean regular nesting areas are genetically very different and until now remained isolated[54], this bilateral colonisation may lead to a novel genetic admixture. This potential admixture can increase the fitness of the offspring because of the hybrid vigor, but it can also produce the opposite effect due to outbreeding depression[55,56]. Consequently, knowing the origin of the nests and assessing the degree of admixture between Atlantic and Mediterranean individuals is crucial to understand the genetic viability of the emerging population, especially considering the high variability found in terms of hatching success. Genomic methods allow for studying migration patterns[57] and population structure[58], thus tracing the origin of the breeding individuals. Unfortunately, all these analyses require the establishment of a baseline by characterising the regular nesting areas with the same markers used in the emerging nesting sites, something not yet available with the genomic markers used in the present study but published for the mtDNA D-loop region analysed[43]. For this, and considering the explosive increase of the nests, a baseline of genomic data built with individuals from different regions is in progress to improve the origin assignment and admixture in future studies.

The recent increase in nesting activity in the Western Mediterranean[28] and the higher production of females, at least in some nests (present study), indicate that we may be at the beginning of the establishment of the new population. Previous research suggested that the new population would start to grow exponentially once females born in the new area start to return due to philopatry and reproduce in different nesting season as remigrants[26]. Consequently, the detection of returning nesting females in different nesting seasons would be indicative that the colonisation in our study area has reached this point. Our MDS results based on 2,291 SNPs, clustered individuals from different nests that could suggest that were laid by the same re-nesting female. Individuals from the nests SP01, SP02 and SP05 clustered together in the MDS and were laid in 2016, 2017 and 2018 respectively. However, we can discard that some of these nests were laid by the same female as different mtDNA haplotypes were found in different nests (CC-A1.1 in SP02 and CC-A2.1 in SP01 and SP05) and were clearly separated by the relatedness analysis (e.g., individuals from different nests exhibited very low relatedness values). In addition, we discarded the presence of remigrant females since none of the individuals analysed clustered with individuals from different years, therefore suggesting that nests were laid by different females. Although, analysing a single individual per nest could be enough to perform a MDS to detect clusters of different nests, the genomic analysis of multiple individuals per nest allowed us to identify an artifact caused by uneven sample size. The MDS with a single individual per nest (Fig. 5b), splits the individuals from nests SP01, SP02 and SP05, especially the individual from nest SP02 with a D-loop haplotype widespread in the Western Atlantic rookeries. Additionally, analysing multiple individuals per nest helps delineating clusters of siblings to the same nest in relation to individuals from other nests with a relatedness analysis. Consequently, the number of related samples that are included when using all data together must be evaluated depending on the analysis performed. Thus, the similarity between related samples and uneven sampling sizes may distort the relationships with the rest of the samples and may affect the interpretation of the results of the MDS as the presence of many related individuals seems to mask the relationships between unrelated ones. So, the number of samples from the same nest or the same area that are included in the analysis when analysing all data together may affect the MDS plot and should be considered in genetic analyses to correctly identify the relationships.

The hatchlings from nests SP07 and SP11 clustered together in the MDS, have the same rare mtDNA haplotype and exhibited high relatedness values to the point that relatedness among individuals from these two nests were indistinguishable from siblings within the same nest. All this evidence supports the fact that only these two nests were laid by the same re-nesting female, the same year and 213 km apart. The exact laying date of nest SP11 is not available, as hatchlings were detected during the emergence phase. However, considering the range of incubation duration in our data, the female laid the two nests in an interval between 14 and 30 days, consistent with the internesting interval in loggerhead turtles which is between 12 and 16 days[59–62]. The detection of a re-nesting female laying more than one nest within the same year is not unprecedented, as previous studies on the loggerhead sporadic events in the Western Mediterranean, found one female laying two nests in 2015 in a 14-day interval and 120 km apart[26]. In regular nesting areas, loggerhead turtle females lay on average 3–5 nests per season with the internesting intervals mentioned above[63]. On green turtles the number of nesting events per season can be even higher and at shorter internesting intervals[25]. Multiple nests per female within the same year are the consequence of the gradual maturation of eggs, and even can be the result of the same mating event[64,65].

Thus, the nests laid by a female within the same year are considered part of the same reproductive season, since usually the female remains in the area or performs short internesting migrations during the whole nesting season[66]. Alternatively, if we had found nests laid by the same female in different years, this would imply that it is a remigrant due to philopatry, which in marine turtles involve long recurrent migrations from breeding to foraging areas[25,67].

While nesting activity is becoming more frequent on the Spanish Mediterranean seaside, our results and those obtained by Carreras et al. 2018 suggest that this increase is not the result of remigrant individuals but an increase of the number of colonisers coming from distant areas. On one hand, both studies concluded that no remigrant was present in all nests analysed within their respective periods. On the other hand, despite both studies used different markers, they both analysed the same region of the D-loop, which is not very informative as common haplotypes are very frequent. Consequently, we cannot rule out that common haplotypes between both studies correspond to the same remigrating female, but different haplotypes are an indication of different nesting females. Considering that the typical remigrant interval of the species is two years, only one nest laid in 2014 had the same haplotype of the nest laid in 2016. On the contrary, no nest laid in 2015 had the same haplotype than the nest laid in 2017. These two-year comparisons suggest that nests among different reproductive seasons were laid by different females with only one potential remigrant across studies (Supplementary table S1). Future studies analysing all the sporadic events with the same methodology are desirable to confirm our hypothesis, particularly if the nest laid in Tarragona in 2014 (N29, CC-A2.1) was not laid by the same female than the nest laid in Sueca in 2016 (SP01, CC-A2.1), but also testing all potential pairs of nests in case there are individuals remigrating at longer intervals in this emerging population. An additional consideration is that the present study includes the nests detected over the four nesting seasons, but some nests may remain undetected and thus unsampled. Consequently, there is still the possibility that remigrants could be present in the unsampled nests. Considering all the evidence together and the potential drawbacks of the study, we propose that the recently raised number of nests may be caused by two non-exclusive hypotheses (Fig. 2). On one hand, the number of females arriving to the Western Mediterranean as potential colonisers may be increasing. This could be favoured by the global increase in the size of the sea turtle regular nesting populations[31] derived from the success of conservation efforts worldwide[68–70]. Furthermore, we are witnessing a feminisation of sea turtle populations[31] triggered by climate change[71] which would drive an increase in the number of females recruiting to the adult population and a rise in the number of nests[72] even with the same total number of adult individuals. Thus, the feminisation of the populations of origin would likewise increase the number of prospective colonising females found nesting in the emerging nesting sites. On the other hand, the increased sea surface temperatures in the Western Mediterranean might be favouring an early maturation of the juvenile or adult females feeding in the neighbouring foraging grounds[33], as SST in feeding areas affects nesting phenology, leading to an earlier onset of nesting[73]. This early maturation would increase the chances of laying a nest in the nearby area, as this advanced maturation is produced before they are able to return to the nesting beaches of origin to reproduce[29]. Previous studies indicated that a minimum sea surface temperature of around 22 °C is needed for gonad maturation[74,75] and 21 °C are needed to initiate the nesting season[34]. Our results show that the spatial and temporal location of the nesting events in the Spanish Mediterranean coast has always been above this threshold during the nesting years. Hence, the combination of the factors described above may be increasing the number of mature females in the Western Mediterranean thus explaining the current explosion of the number of clutches.

Our results confirm that we are witnessing a shift of species distribution at evolutionary level induced by climate change. Conservation measures are essential to help the growing population. First, monitoring the different approaches of this event is needed to evaluate the effects of rising temperatures on sex ratio, fitness, viability, and hatchling survival. Second, as emerging nests are occurring on anthropized beaches, analysing the human impact on the potential nesting beaches is essential to ensure the minimal anthropic disturbance. For instance, the effects of light pollution[76] or the coastal erosion from massive urbanization[77]. Furthermore, education and awareness are fundamental parts of this framework. Citizens play a key role in the detection of the nests, which is substantial to obtain samples and biological data and ultimately, enabling management. Indeed, this study has added significance by becoming a pilot project for future colonisation events in migratory species influenced by human pressures. Translation of scientifically based monitoring to proactive conservation measures could facilitate the expansion and viability of the species in a warming world. The detection and study of these new events through extensive genomic monitoring and study on potential suitable habitats, coupled with its protection and conservation may be crucial to facilitate the possible expansion and long-term survival of the species.

Methodology

Sampling and data collection

Data and samples were obtained from loggerhead sea turtle nests laid on the Spanish coast between 2016 and 2019 (Table 1). When a nesting event occurred, we collected the basic data, previously published[28]. In addition, we estimated the minimum and maximum percentage of female offspring using the incubation duration and applying four different models[78–81]. We also gathered information on the habitat suitability for nesting[41] and the present and future hatching success[42] from published map models following the same procedures of previous studies[26]. Although the study species is listed in CITES, transportation of samples within the same country does not require CITES permits. As part of the national and regional management plan of the nesting events, some of the hatchlings are routinely kept for one year as part of a headstarting program to increase their first-year survival in the Foundation for the Conservation and Recovery of Marine Animals in Barcelona (CRAM), the Oceanografic of Valencia or the Palmaquarium Foundation of the Balearic Islands. Taking advantage of this management action, we obtained blood samples from headstarted individuals from some of the nests (SP04; SP07; SP08; SP09; SP11) taken at approximately one year of age as part of their routinely veterinary check. Approximately, 100 µl of

blood was extracted from the cervical sinus following standard procedures[82]. Additionally, muscle or skin samples were collected from dead hatchlings or embryos found during the nest excavation after natural emergence of hatchlings. Both blood and tissue samples were fixed and stored in 96% ethanol at − 20 °C.

Laboratory procedures

Genomic DNA was extracted using the Puregen Kit (Qiagen) following the manufacturer's protocol and suspended in 25 µl of Elution Buffer, the DNA concentration and quality was measured with Nanodrop. We considered one sample per nest for sequencing an 800-bp fragment of the mtDNA D-Loop region[83]. Each reaction was prepared in a final mix volume of 15 µl containing 5.08 µl of Nuclease Free-water (Thermo Scientific), 3 µl of PCR Buffer 5X (GoTaq Promega), 1.8 µl of dNTPs (1 mm), 0.6 µl of $MgCl_2$ (25 mm), 1 µl of Forward primer (10 µm), 1 µl of Reverse primer (10 µm), 0.12 µl of Gotaq G2 Flexi DNA Polymerase (Promega 5U/µl), and 2 µl of DNA (~ 10 ng/µl), the amplified was verified in a 1% agarose gel. Next, 3 µl of the amplified product were purified with 2 µl of ExoSAP (0.4 U of EXO and 0.4 U of TSAP). Later, 1 µl (5 µm) of Forward primer was added to the purified product and dried at 80 °C for 30 min. We used only the Forward primer, as it was sufficient to recover the entire ~ 800 bp sequence used for haplotype delimitation for comparison in public databases. Finally, the amplification was sequenced on an ABI 3730 automated DNA analyser (Applied Biosystems) at the Seveis Cientificotècnics from the University of Barcelona.

To perform a 2bRAD genotyping, we analysed a variable number of individuals per nest (Table 1), depending on the availability of samples. We constructed individual libraries digesting 180 ng of DNA (~ 40 ng/µl) with AlfI enzyme using the protocol from Barbanti et al. (2020)[38]. The quantity and concentration used maximizes the total number of sequences recovered, as shown in previous studies[39]. For this study, we used selective-adaptors (5'-WN-3') that reduce the number of analysed markers without compromising genetic differentiation[84]. After digestion, ligation and DNA amplification, the quality of the amplified fragment (~ 165 bp) was verified in a 1.8% agarose gel. The successful amplified product was purified using magnetic beads (SPRIselect) to remove primers and fragments longer and shorter than 165 bp. The DNA concentration of the purified libraries were measured with the Quant-iT™ Picogreen dsDNA Assay Kit (Thermo Fisher Scientific) and pooled calculating ~ 180 ng of DNA per sample. The pool was sequenced with a HiSeq 2500 Ilumina at the Centre for Genomic Regulation (CRG).

Data filtering and genotyping

We processed the 2bRAD sequences using customized scripts[39], trimming the raw sequences to remove ligation adaptors and cutting all the fragments to the same length (34 bp). We mapped the trimmed sequences to the published reference genome of the loggerhead turtle (GenBank accession GCA_023653815.1)[85] using Hisat2-2.2.1[86], to identify polymorphic nucleotides (SNPs) with BCFtools[87]. Individual genotypes were outputted as SNPs in a VCF file. We filtered our data using VCFtools[88], by removing individual genotypes based on less than five reads. Loci with a mean depth above 50 (which corresponds to the upper whisker defined as 1.5 times the interquartile range from the data) and loci present in less than < 95% of the individuals were removed.

The D-Loop sequences were aligned, cut and blasted with published haplotype sequences found in the database maintained by the Archie Carr Center for Sea Turtle Research (https://accstr.ufl.edu/) using BIOEDIT[89]. We identified the regular nesting region in which each haplotype was found following the haplotype frequencies found in previous studies[43,90] to determine the potential origin of the nesting females that laid each nest.

Genetic diversity and relatedness

The percentage of polymorphic loci and observed heterozygosity per nest were used as a measure of relative genetic diversity. The percentage of polymorphic loci per nest was calculated using GENALEX 6.503[91] and the observed heterozygosity was obtained using the function '–het' of VCFTOOLS. Relatedness among individuals was calculated based on the number of alleles shared between pairs of samples by applying the '–relatedness' statistic function of VCFTOOLS based on the Manichaikul et al.[44] method. We used these relatedness values to create a heatmap and a dendrogram using the function ggplot of 'ggplot2'[92] in R program vs 4.1.1[93]. Finally, we used PLINK vr. 1. 07[94] to perform a Multidimensional Scaling Analysis plot (MDS) considering the Identity By State (IBS) individual pairwise distance and the first 3 dimensions were plotted using the function scatterplot3d of 'ggplot2'[92] in R program vs 4.1.1[93]. Likewise, we also randomly selected one individual from each relatedness cluster, to plot an additional three-dimensional MDS.

SST during nesting period

Western Mediterranean SST values were obtained from E.U. Copernicus Marine Service Information (doi.org/https://doi.org/10.48670/moi-00173) for the months of June and July in the years when nesting or attempted nesting occurred between 1990 and 2019. These months were selected following the same rationale than previous studies[34] since the nesting in the western Mediterranean is concentrated around this period (Table S1), and it has been suggested that a minimum of 21 °C of SST is needed to initiate nesting[29,34]. For each year, we obtained the mean daily SST of the two months considering two separated areas, on one hand the Balearic Sea (42.6 N, 39.1S, 4.2 E, − 0.5 W) and on the other the Algerian Sea (38.9 N, 34.8S, 6.6 E, − 2.4 W) as these areas are used by individuals foraging in the Western Mediterranean[53,95,96]. The daily mean SST for each area was averaged for every year using map algebra with the QGIS software vs 3.22.9. We used a Spearman's rank correlation test (as a non-parametric analysis) to evaluate if the SST mean temperatures were correlated to the annual number of events, using the cor function as implemented in R program vs 4.1.1[93]. Finally, the fine scale SST spatial distribution of years with nesting events were graphically plotted through QGIS vs 3.22.9 software.

Data availability

D-loop haplotypes accession numbers are given in the results (CC-A1.1 EU179436; CC-A2.1 EU179445; CC-A3.1 EU179455; CC-A31.1 AM949678) and also can be found in the database maintained by Archie Carr Center for Sea Turtle Research (https://accstr.ufl.edu/). 2bRAD raw data can be found at the European Nucleotide Archive (ENA) project PRJEB64665. Data on the nesting events can be found in the Supplementary material.

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Author contributions

C.C. and M.P. conceived and designed the study. C.C., E.A., J.L.C.P., F.E., G.F., S.G. and J.T. obtained the samples and in situ data from the sporadic nests. A.L.O. did the laboratory procedures. A.L.O., G.M.C., C.C., M.P. and C.P. did the data analyses. G.M.C. did the sea surface temperature analysis and the illustrations of the manuscript. A.L.O., G.M.C., M.P., C.P. and C.C. wrote the manuscript with input and review from all the authors.

Competing interests

The authors declare no competing interests.

Additional information

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SUPPLEMENTARY MATERIAL

SUPPLEMENTARY MATERIAL

https://drive.google.com/drive/folders/1f7u4QwntxOEbc_qp7ujrw9Pf752_uJ7L?usp=sharing

Supplementary Tables

Nest code	Year	Beach (Location)	Description	Latitude [°N]	Longitude [°E]	Laying date	Emergence date	*Eg*	*Hs [%]*	*Id*	*Of [%]*	Habitat	Predicted Hs				Haplotype	Reference
													1950 - 2000	2020	2050	2080		
N1	1870	Mar Menor	Possible nest	37.771	-0.786	-	1870	-	-	-	-	Good	35,9	(+)10	(+)5	(+)5	-	Carreras et al. 2018
N2	1990	Ebro delta	Dead embryo	40.655	0.789	-	09/1990	-	-	-	-	Moderate	21,7	(+)10	(+)10	(+)10	-	Carreras et al. 2018
N3	2001	Vera	Full nest	37.222	-1.802	-	27/07/2001	97	43,3	58	65_22_72_10	Good	35,9	(+)10	(+)10	(+)5	CC-A3.1	Carreras et al. 2018
N13	2006	Puzol	Full nest	39.607	-0.265	-	11/08/2006	>78	36,8	>50	-	Moderate	21,7	(+)10	(+)10	(+)10	CC-A2.1	Carreras et al. 2018
N14	2006	Premià de Mar	Full nest	41.488	2.357	-	27/10/2006	82	68,3	-	-	Marginal	14,5	(+)10	(+)10	(+)10	CC-A1.1	Carreras et al. 2018
N21	2011	Malgrat de Mar	Full nest	41.645	2.752	-	01/10/2011	134	70,8	-	-	Marginal	14,5	(+)10	(+)10	(+)10	CC-A2.1	Carreras et al. 2018
N27	2014	Alicante	Full nest	38.376	-0.409	30/06/2014	28/08/2014	131	79	59	57_12_52_3	Moderate	21,7	(+)10	(+)10	(+)10	CC-A20.1	Carreras et al. 2018
N28	2014	Tarragona	Full nest	41.119	1.276	31/08/2014	-	89	0	-	-	Marginal	14,5	(+)10	(+)10	(+)10	CC-A3.1	Carreras et al. 2018
N29	2014	Tarragona	Full nest	41.127	1.296	-	30/10/2014	58	-	-	-	Marginal	14,5	(+)10	(+)10	(+)10	CC-A2.1	Carreras et al. 2018
N32	2015	Torrevieja	Full nest	38.015	-0.653	31/07/2015	09/10/2015	85	32,5	53,5	89_87_99_99	Moderate	21,7	(+)10	(+)10	(+)10	CC-A2.1	Carreras et al. 2018
N33	2015	Pulpí	Full nest	37.345	-1.684	17/07/2015	09/09/2015	81	52,9	69	5_0_0_0	Good	35,9	(+)10	(+)10	(+)10	CC-A2.1	Carreras et al. 2018
SP01	2016	Les Palmeres (Sueca)	Full nest	39.256	-0.262	03/07/2016	05/09/2016	88	54	63	26_0_4_0	Excellent	76.9	(+)5	(-)5	(-)10	CC-A2.-1	Present study

SP02	2017	Migjorn (Peñiscola)	Hatchlings on beach	40.358	0.403	-	11/10/2017	-	-	-	-	Moderate	21,7	(+)10	(+)10	(+)10	CC-A1.-1	Present study
SP03	2018	Sant Simó (Mataró)	Full nest	41.541	2.459	15/06/2018	08/08/2018	173	56,1	54	87_85_98_99	Marginal	14,5	(+)10	(+)10	(+)10	-	Present study
SP04	2018	La Descàrrega (Premià de Mar)	Full nest	41.486	2.349	01/08/2018	28/09/2018	62	93,1	58	65_22_72_10	Marginal	14,5	(+)10	(+)10	(+)10	CC-A3.1	Present study
SP05	2018	Vilafortuny (Cambrils)	Full nest	41.069	1.099	-	16/09/2018	112	80,4	-	-	Marginal	14,5	(+)10	(+)10	(+)10	CC-A2.-1	Present study
SP06	2018	Ardiaca (Cambrils)	Hatchlings on beach	41.054	1.028	-	24/09/2018	-	-	-	-	Marginal	14,5	(+)10	(+)10	(+)10	-	Present study
SP07	2019	Del Serradal (Castellón de la Plana)	Full nest	40.005	0.032	13/07/2019	14/09/2019	112	65	63	26_0_4_0	Moderate	21,7	(+)10	(+)10	(+)10	CC-A31.-1	Present study
SP08	2019	D'en Bossa (Sant Jordi de ses Salines)	Full nest	38.884	1.406	25/07/2019	10/09/2019	58	65,5	46	100_100_100_100	Good	21,7	(+)10	(+)10	(+)10	CC-A2.-1	Present study
SP09	2019	Calblanque (Cartagena)	Full nest	37.598	-0.750	28/07/2019	18/09/2019	69	30,4	52	93_96_100_100	Excellent	35,9	(+)10	0	(-)10	CC-A2.-1	Present study
SP10	2019	D'es Cavallet (Sant Francesc de s'Estany)	Full nest	38.841	1.403	29/07/2019	-	102	0	-	-	Marginal	21,7	(+)10	(+)10	(+)10	-	Present study
SP11	2019	Castelldefels (Castelldefels)	Hatchlings on beach	41.264	1.957	-	06/10/2019	-	-	-	-	Marginal	14,5	(+)10	(+)10	(+)10	CC-A31.-1	Present study

Supplementary table 1. Sporadic nests of loggerhead turtle in the Spanish coast from 1870 to 2019 and data collected. *Eg:* number of eggs ; *Hs*: hatchling success (%); *Id*: Incubation duration (days); *Of:* percentage of female offspring using *Id* and calculated from different models[1–4]; Habitat: predicted habitat suitability for loggerhead turtles as estimated from MaxEnt models for the Mediterranean region[5]; Predicted Hs: Predicted hatchling success at different years as predicted in published models[6], the baseline value corresponds to the period between 1950 and 2000 and the rest of values indicate the expected percentage of increase or decrease respect the baseline values.

Attempt code	Location	Latitude [°N]	Longitude [°E]	Year	Attempt type
A1	Delta del Ebro	-	-	1972-73	Track
ES_C01	Girona	41.673	2.793	2014	Female
ES_C02	Barcelona	41.613	2.653	2014	Female
ES_C03	Almeria	36.939	-1.934	2014	Female
ES_C04	Ibiza	38.098	1.5540	2015	Female
ES_C05	Ibiza	38.983	1.5347	2015	Female
ES_C06	Ibiza	38.983	1.534	2015	Female
ES_C07	Murcia	37.672	-0.728	2015	Female
ES_C08	Murcia	37.4018	-1.591	2015	Female
ES_C09	Murcia	37.599	-0.7502	2016	Track
A2	Castelldefels	41.281	2.088	2016	Female
ES_C10	Almeria	37.247	-1.768	2016	Female
ES_C11	Alicante	38.355	-0.431	2016	Female
ES_C12	Ibiza	38.901	1.421	2017	Female
ES_C13	Barcelona	41.585	2.580	2017	Female
ES_C14	Alicante	38.054	-0.651	2017	Track
ES_C15	Murcia	37.700	-0.739	2017	Female
ES_C16	Murcia	37.599	-0.750	2017	Female
ES_C17	Murcia	37.599	-0.7502	2017	Track
ES_C18	Amposta	40.654	0.801	2017	Track
ES_C19	Murcia	37.598	-0.751	2017	Track
ES_C20	Murcia	37.680	-0.732	2017	Female
ES_C21	Valencia	39.513	-0.319	2018	Female
ES_C22	Castellón	40.042	0.060	2018	Female
ES_C23	Altea	38.630	-0.006	2018	Female
ES_C24	Orihuela	37.917	-0.719	2018	Female
ES_C25	Alicante	37.871	-0.754	2018	Female
ES_C26	Barcelona	41.284	2.0960	2018	Female
ES_C27	Murcia	37.597	-0.762	2019	Track
ES_C28	Tarragona	40.68	0.852	2019	Track
ES_C29	Murcia	37.718	-0.7407	2019	Track
ES_C30	Murcia	37.718	-0.7407	2019	Track

Supplementary Table S2. List of sporadic loggerhead turtle nesting attempts on the Mediterranean Spanish coast from 1972 to 2019. Female indicates that an adult female was found on the beach but no related nest was found. Track indicates that turtle tracks were found on the beach but without evidences of a nest. Data obtained from Hochscheid et al. (2022) [7], except A1[8] and A2 (present study).

Nest Code	Beach (Locality)	Individual Code	Ho	Raw reads	Filtered reads	Mapped reads [%]	Number of loci
SP01	Les Palmeres (Sueca)	SP01_1	0.252	5,381,655	4,609,524	95.4	2290
		SP01_2	0.260	3,268,581	2,811,413	95.5	2277
SP02	Migjorn (Peñiscola)	SP02_1	0.257	6,380,033	5,465,033	95.5	2291
		SP02_2	0.240	5,927,855	5,169,399	95.0	2290
SP04	La Descàrrega (Premià de Mar)	SP04_1	0.252	6,386,869	4,126,609	95.5	2273
		SP04_2	0.250	7,474,811	6,109,951	95.3	2291
		SP04_3	0.267	9,888,986	8,091,694	95.6	2290
		SP04_4	0.251	7,380,964	5,910,627	94.4	2290
		SP04_5	0.257	8,538,495	6,761,385	95.5	2291
		SP04_6	0.255	8,279,236	7,052,296	95.7	2291
		SP04_7	0.261	8,698,409	7,428,122	95.7	2291
		SP04_8	0.263	4,320,601	3,885,076	95.7	2281
SP05	Vilafortuny (Cambrils)	SP05_1	0.281	3,823,190	3,319,194	91.3	2289
		SP05_2	0.324	8,279,366	6,880,997	74.4	2291
		SP05_3	0.259	6,473,971	5,672,384	95.7	2290
		SP05_4	0.251	5,258,447	3,512,435	57.9	2221
SP07	Del Serradal (Castellón de la Plana)	SP07_1	0.251	4,070,232	2,698,674	95.9	2288
		SP07_2	0.269	4,784,831	3,797,112	95.7	2291
		SP07_3	0.264	3,865,348	3,017,269	95.5	2290
		SP07_4	0.280	6,083,117	5,090,768	94.3	2202
		SP07_5	0.259	4,316,300	3,473,349	95.9	2288
		SP07_6	0.249	4,654,665	3,103,020	95.7	2290
		SP07_7	0.263	5,030,768	4,097,368	94.2	2006
SP08	D'en Bossa (Sant Jordi de ses Salines)	SP08_1	0.265	4,660,832	3,439,416	95.6	2291
		SP08_2	0.252	5,754,139	4,527,912	95.6	2291
		SP08_3	0.269	4,914,376	4,092,821	93.9	2079
		SP08_4	0.264	4,511,422	3,782,799	93.8	2006
		SP08_5	0.265	4,244,291	3,288,598	95.4	2289
		SP08_6	0.299	6,187,107	5,254,976	93.8	2200
		SP08_7	0.277	4,768,676	4,047,806	93.8	2030
SP09	Calblanque (Cartagena)	SP09_1	0.255	5,549,998	4,524,128	95.9	2291
		SP09_2	0.251	5,175,547	3,967,852	95.7	2291
		SP09_3	0.263	5,065,870	4,032,402	93.0	2042
		SP09_4	0.279	5,231,907	3,950,012	94.0	2002
		SP09_5	0.258	4,288,713	3,373,797	95.5	2287
		SP09_6	0.256	3,950,197	3,693,181	95.6	2289
		SP09_7	0.259	4,491,761	3,579,951	95.7	2291
SP11	Castelldefels (Castelldefels)	SP11_1	0.246	3,039,933	2,615,349	95.0	2282
		SP11_2	0.244	6,505,721	5,299,574	95.0	2291
		SP11_3	0.248	5,381,471	4,306,982	95.9	2291

		SP11_4	0.245	4,270,152	3,544,631	95	2290
		SP11_5	0.267	2,426,253	1,297,965	95.8	2073
		SP11_6	0.248	5,468,171	3,958,123	95.9	2291
		SP11_7	0.247	5,549,441	4,455,992	95	2291
		SP11_8	0.267	6,478,317	5,353,343	95.9	2291

Supplementary table 3. Individuals genotyped and associated genomic data of the 45 individuals. *Ho*: individual observed heterozygosity; Filtered reads: number of trimmed sequences; Mapped reads: percentage of reads mapped against the reference genome; Number of loci: number of polymorphic loci per individual.

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CHAPTER 3.3

INDIVIDUAL GENOMIC ASSIGNMENT OF LOGGERHEAD SEA TURTLE IN THE EMERGING NESTS IN SPAIN

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ABSTRACT

The loggerhead sea turtle (*Caretta caretta*) is currently colonizing the Western Mediterranean as a consequence of climate change, shifting towards more favorable areas. Historically, loggerhead nesting in the Mediterranean was restricted to the Eastern basin, there has been a significant increase in nesting events in the Western basin. Climate models predicted that by 2020, the Spanish Mediterranean coastline would become suitable for regular nesting, a prediction that has been corroborated by the observed increase in these events in recent years. One of the key aspects is to identify the origin of breeders to understand which populations are the source of this colonization. To do so, we used different genomic baselines to assign 45 hatchlings from 8 nests laid between 2016 and 2019 on the Spanish Mediterranean coast to different population levels. Globally, using the RMU Baseline-R (4,321 SNPs), we assigned each individual to three Regional Management Units (RMUs): North West Atlantic (NWA), North East Atlantic (NEA), or Mediterranean (MED). The results revealed that most of the individuals had a MED RMU origin, while only two individuals from the same nest showed a hybrid origin between Atlantic and Mediterranean RMUs. Within the Mediterranean, we used the MED Baseline (5,177 SNPs) to assign individuals to one of three SubRMUs: Greece (GRE), Levantine (LEV), or Sirte (SIR). A significant proportion of individuals were related to the Greece SubRMU, while others exhibited mixed origins. This suggests the possibility of mating between individuals from different origins, potentially facilitated by the ongoing colonization process. Additionally, using the RMU Baseline (5,308 SNPs), we evaluated five genetic clusters: NWA, NEA, MED, GRE, LEV, and SIR and identified the nest with mixed origin from the NWA and MED RMUs, to be sired by a NWA female and a male from SIR. The findings also highlighted the potential presence of multiple paternities within some nests, a phenomenon not uncommon in this species. The use of genomic tools enabled high-resolution individual assignments. Finally, we emphasize the importance of continuous monitoring to better understand the temporal patterns of colonization and the dynamics of these emerging populations.

INTRODUCTION

The global climate is changing at an unprecedented rate, so species are facing environmental pressures in their regular ranges (Loarie et al., 2009). In response, species have started to alter their phenology, shift their geographic distribution, and modify their trophic interactions (Dalleau et al., 2012). Species can respond to climate change through at least three different but not exclusive mechanisms: (1) phenotypic plasticity, (2) shifts in allele composition through natural selection, and (3) changes in distribution areas (Fuentes et al., 2020; Waldvogel et al., 2020).

The capacity of species to adapt to global warming by dispersing to more favorable regions may be constrained by extrinsic factors such as the presence of biogeographic barriers, as well as intrinsic factors like the tendency to reproduce at natal sites, known as philopatry (Malhi et al., 2022).

The life history of sea turtles is closely associated with both water and sand temperatures, making them vulnerable to global warming (Tanner et al., 2019). Adaptative responses in reproductive phenology, nest depth, and nesting site selection may help them mitigate the effects of rising temperatures; however, evolutionary changes may occur too slowly to keep pace with the rapid global warming (Abella Perez et al., 2016).

Potential mechanisms for rapid adaptation could be a shift in nesting beaches, as philopatry in sea turtles is flexible, allowing them to occupy beaches separated by hundreds of kilometers, even within the same season (Barbanti et al., 2022; Stewart et al., 2014). If so, the shift would be gradual towards beaches with the optimal temperature range (Martins et al., 2020).

The colonization of new nesting beaches is an alternative process that has occurred in the past. In a scenario of rising sea temperatures, early adults that remain in feeding

areas may mate and nest on nearby beaches if the thermal conditions support gonadal development, oviposition, and embryo development. This could explain the occurrence of nesting events outside the usual range and may eventually lead to the establishment of new populations (Carreras et al., 2018), Chapter 3.2.

Historically, the nesting of loggerhead turtles (*Caretta caretta*, Linnaeus 1758) in the Mediterranean Sea has been restricted to the Eastern basin (Casale et al., 2018). Although there had been a few sporadic nesting events in the Western Mediterranean as old as 1870, where low sand temperatures inhibited regular nesting in the past. However, since 2001, there has been a steady increase in the detection of nesting activities (including false crawls, nests, and hatchlings) along the Spanish coast and in the whole Western Mediterranean (Hochscheid et al., 2022).

Climate modeling had predicted that by 2020, the Spanish Mediterranean coasts, along with the entire European coast of the Western Mediterranean, would become suitable for regular loggerhead turtle nesting (Pike, 2014). The observed increase in nesting events appears to support this prediction, coupled with recent studies showing that nesting in the past was unlikely as the conditions for viable nesting were not favorable (Cardona et al., 2023) .

The hypothesis that these nesting events are remnants of a nearly extinct population due to urbanization has been dismissed (Carreras et al., 2018). Instead, it is hypothesized that this represents a new colonization event, likely driven by global warming and improved thermal conditions on the Spanish coasts conducive to successful turtle nesting (Cardona et al., 2023). Recent studies have indicated that the nesting events recorded between 2016 and 2019 are independent, with no evidence of remigrant females; however, genomics has identified two nesting events by the same female within the same season (Chapter 3.2). Given the consistent increase in nesting observed

each season, long-term genomic studies are essential, not only to understand the species adaptation mechanisms but also to determine the stage of establishment of a new resident population. One of the key aspects is to identify the origin of breeders to understand which populations are the source of this colonization. Previous studies suggested that this colonization is sustained by individuals from the Atlantic and the Mediterranean (Carreras et al., 2018) but relied on markers with low resolution, including a fragment of the D-loop of the mtDNA and 7 microsatellites. Confirming this bilateral colonization is crucial since it would imply that the new nesting events can produce an admixture of RMUs, suggested to be genetically isolated (Carreras et al., 2011), leading to potential outbreeding depression that could compromise the development of this emerging population. The application of genomic tools in the species (Barbanti et al., -, in prep) and the development of a baseline to perform Individual Assignments (Chapter 3.1) open the possibility, for the first time, to assess the origin of the nesting events studied with the same methodology (Chapter 3.2) with high resolution and at a fine scale. Consequently, the aim of this work is to infer the origin of the 45 hatchlings of the 8 nests from 2016-2019 (Chapter 3.2) combining their genotypes with the baseline information of the North Atlantic and Mediterranean populations following the best methodology, according to the hierarchical analysis proposed in Chapter 3.1.

METHODOLOGY

Sampling and Data Collection

We analyzed the 45 individuals of the 8 nests laid on the Spanish coast between 2016 and 2019 using the 2bRAD sequences presented in Chapter 3.2, and the sequences of the baseline data presented in Chapter 3.1 mapped to the reference genome (GenBank accession GCA_023653815.1) (Chang et al., 2023).

Following the procedure developed in Chapter 3.1 to perform individual assignments on new unknown samples, all samples (all baseline and unknown samples) were genotyped

simultaneously with the joint genotyping strategy to recover the SNPs representing the genetic diversity of the baseline also in the unknown samples. Genotyping was performed with BCFtools (Li, 2011). Individual genotypes of the unknown individuals were outputted as SNPs in a VCF file and the list of SNPs of all baseline datasets, developed in Chapter 3.1, were extracted from the file with the unknown individuals using the '–snps' function in VCFtools (Danecek et al., 2011). Consequently, for each level of individual assignments (RMU Baseline, RMU Baseline-R and MED Baseline), a file of the genotypes of the 45 unknown individuals was obtained with the same SNPs as the corresponding Baseline file (RMU Baseline: 5,308 SNPs, RMU Baseline-R: 4,321 SNPS and MED Baseline datasets: 5,177 SNPs).

Individual assignment

We used the baseline information reported in Chapter 3.1 to perform the Individual Assignments of the unknown samples using the assignPOP v1.1.4 R package (Chen et al., 2018) and considering the hierarchical approach reported in the same Chapter. We used the SVM model to analyze the probability percentage for each of the genetic clusters, as it has been shown to be the best model to perform IAs in the species (Chapter 3.1). Thus, firstly we performed IAs at the global Regional Management Units level considering the potential RMUs of origin: 1) North West Atlantic (NWA), 2) North East Atlantic (NEA) and 3) Mediterranean (MED) using the RMU Baseline-R (n=84). Then, we evaluated the assignment at the SubRMU level using the MED Baseline with all the individuals assigned to the MED RMU and, considering three SubRMUs: 1) Greece (GRE), 2) Levantine (LEV) and 3) Sirte (SIR). In both cases, given that running the same program multiple times did not show significant differences between tests (Chapter 3.3), here we performed a single test using the 'assign.X' function at each level of assignment.

Finally, we performed additional IAs but analyzing both RMU and SubRMU levels at the same time to detect different levels of admixture among RMUs and SubRMUs. To

do so, we used the RMU Baseline dataset but considering 5 genetic clusters: 1) NWA RMU, 2) NEA RMU, 3) GRE SubRMU, 4) LEV SubRMU and 5) SIR SubRMU.

RESULTS

When performing the IAs at RMU level, of the 45 nesting individuals from the 2016 to 2019 nests by comparison with RMU Baseline-R, we identified that all individuals except 2 from the nest SP02 were assigned to the Mediterranean RMU with an assignment probability >0.91 (Fig. 1). These two individuals were assigned to the MED RMU with a probability of 0.6 and 0.5, distributing the rest of the assignment first in the NWA (0.2-0.3) and secondly in NEA (0.1). Thus, the nest SP02 seems a potential hybrid between different RMUs.

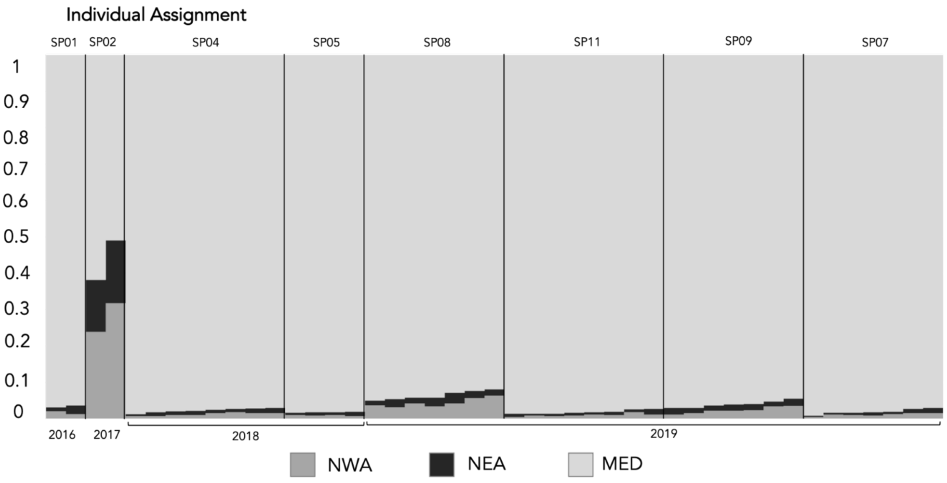

Figure 1: RMU-level assignment probabilities of the 45 individuals from the 8 nests laid in Spain from 2016 to 2019 using the RMU Baseline-R dataset. Each bar represents the assignation probability of one individual to belong to a RMU (NWA=North West Atlantic, NEA=North East Atlantic and MED=Mediterranean), obtained with assignPOP.

When assigning the 45 hatchlings within the MED RMU using the MED Baseline (Fig. 2), a total of 22 individuals were assigned with a probability 0.7 to GRE, while 8 were assigned to LEV (>0.7) and 2, the two hatchlings from nest SP02 to SIR (>0.7). The other 13 individuals were considered to be of admixed origin, from which 10 individuals showed an admixed origin between GRE and LEV SubRMUs, and the other 3 showed an admixed origin among the 3 SubRMUs (GRE, LEV and SIR).

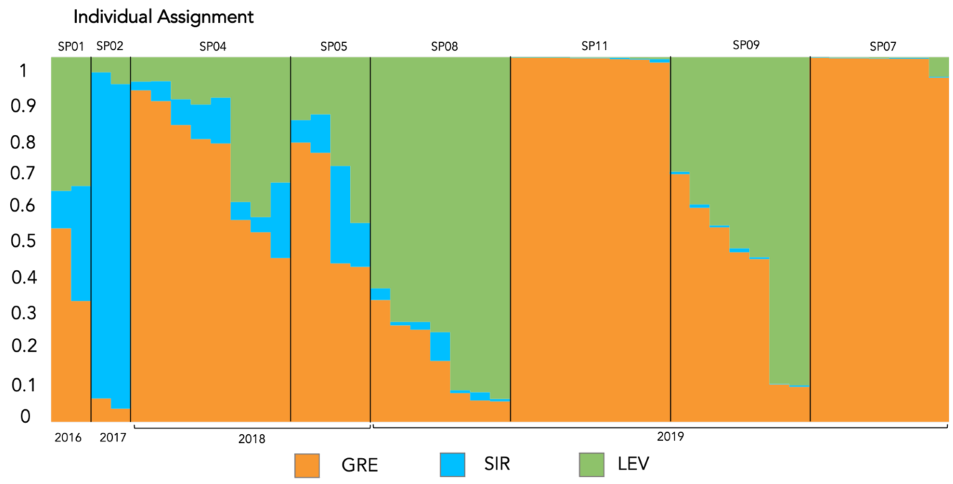

Figure 2: SubRMU-level assignment probabilities of the individuals in nests laid in Spain from 2016 to 2019 using the MED Baseline dataset. Each bar represents the assignation probability of one individual to belong to a SubRMU (GRE=Greece, SIR=Sirte and LEV=Levantine), obtained with assignPOP.

Finally, when considering 5 genetic clusters, 2 of them corresponding to the Atlantic RMUs and 3 clusters to assess the SubRMUs within the Mediterranean Sea using the RMU baseline, the results matched the combination of the two previous analyses. Consequently, two purely GRE nests (SP07 and SP11) were detected, one nest with hatchlings with high probabilities from LEV (SP08), four with different levels of admixture of LEV and GRE (SP01, SP04, SP05 and SP09), three of them with some individuals with some input from SIR (SP01, SP04 and SP05) Finally, with the baseline including the 5 clusters, we were able to recover in nest SP02 a relevant probability of assignment to the NWA RMU as observed previously but also with the Mediterranean component mostly attributed to SIR.

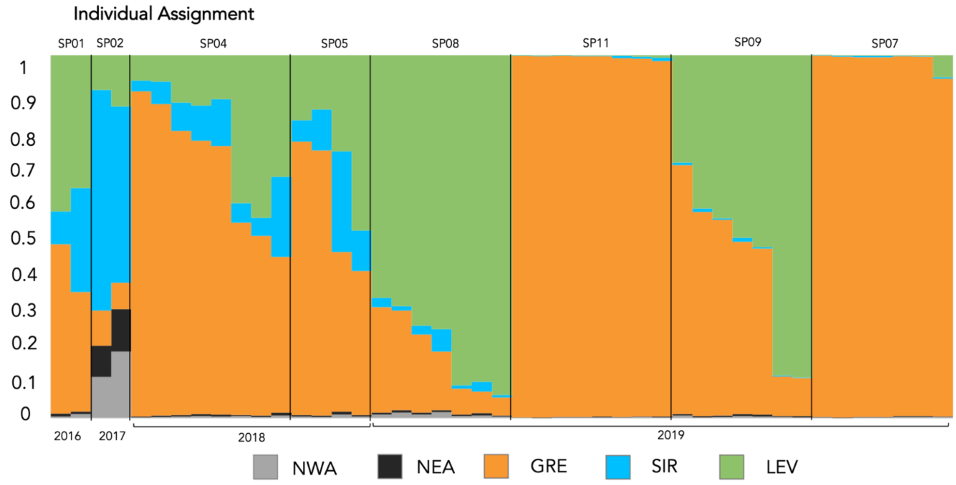

Figure 3: Combined RMU and SubRMU assignment probabilities of the individuals in nests laid in Spain from 2016 to 2019 using the RMU Baseline dataset. Each bar represents the assignation probability of one individual to belong to the RMUs (NWA=North West Atlantic, NEA=North East Atlantic) or to the SubRMU (GRE=Greece, SIR=Sirte and LEV=Levantine) within the Mediterranean RMU, obtained with assignPOP

DISCUSSION

The colonization of the Western Mediterranean as a nesting area for the loggerhead sea turtle is an extraordinary example of “evolution in action” that demonstrates that the species has potential to respond to the global warming threat. Although previous studies have already indicated that this colonization originates from distant Regional Managements Units, the limitations of these methods based on mtDNA and a few microsatellite loci to assign individuals to their nesting population of origin had undermined the potential to assess this origin in detail and to unveil potential processes of admixture that could lead to outbreeding depression. Here, we have used genomics and applied the methods presented in Chapter 3.1 to identify at an individual level, the origin of the turtles sampled in the emerging nests of *Caretta caretta* on the coasts of Spain in Chapter 3.2.

One of the most evident results is that genomic based IAs outperformed past approaches, both in terms of the number of successful assignations but also in the level of detail provided. The use of mitochondrial DNA for Individual Assignments is limited by the

overabundance of common haplotypes even across RMUs, thus making it impossible to determine the origin of most of the individuals using this molecule alone. For instance, only three of the nests in this study presented a haplotype not shared by the three RMUs, the nest SP02 presenting the Atlantic CC-A1 haplotype (present on both NWA and NEA RMUs) and the nests SP07 and SP11 presenting the Mediterranean CC-A31.1. The use of microsatellites improved power of assignation, with 13 out of 18 nests assigned in a study covering the 2001-2015 period (Carreras et al., 2018). In the present work, the genomic based method developed in a previous study (Chapter 3.1) was able to not only determine the origin of all the 8 nests, but also to provide much more detailed information. For instance, all the individuals from the two nests with a Mediterranean exclusive haplotype in previous studies, also known to be laid by the same female (Chapter 3.2), were assigned, not only to the Mediterranean RMU but also to the SubRMU of Greece. Interestingly, the haplotype CC-A31.1 has been found in the Greek nesting population of Kyparissia and in the nesting population of Calabria (Garofalo et al., 2009) which agrees with our results.

Perhaps one of the most interesting results is the assignation of the nest SP02, which presented an Atlantic haplotype but resulted in being a hybrid between Atlantic and Mediterranean RMUs. Combining information of the maternal inherited DNA (Chapter 3.2) and the genomic IA, we can infer that a NWA female reproduced with a male from Libia, being the breeders of nest SP02. Reproduction between Atlantic and Mediterranean individuals is not totally unprecedented, as one nest laid in 2006 was also considered to be hybrid using microsatellites (Carreras et al., 2018). Previous studies using mitochondrial DNA and microsatellites revealed that Atlantic and Mediterranean RMUs were highly differentiated and thus could be considered genetically isolated (Carreras et al., 2011). Recent results using thousands of genomic markers confirmed the complete isolation of the different RMUs at individual level (Chapter 3.1). Finally, from the 103 juvenile individuals foraging in the Mediterranean (Chapter 3.1), none was detected as a hybrid between different RMUs. It is known that Atlantic and Mediterranean RMUs have

clear differences in terms of morphology (Tiwari & Bjorndal, 2000) and development (Piovano et al., 2011). Consequently, the ongoing colonization process in the Western Mediterranean could be favoring the hybridization between RMUs that have been isolated for a long time and potentially accumulating different adaptive polymorphisms. The admixture of isolated populations showing morphological differences has been identified with a high risk to produce outbreeding depression, with an associated loss of fitness of hybrid individuals. Unfortunately, we do not have information on hatchling success of nest SP02 since only hatchlings in the beach were found (Chapter 3.2). Future studies using our methodology to detect hybrids combined with biological information on their fitness, such as hatchling viability or morphological anomalies, are needed to determine potential outbreeding depression in the Western Mediterranean rising nesting populations. The detection of some nests with low success in the area may be indicative of these deleterious effects (Hochscheid et al., 2022).

In this work, at the RMU level, nearly all individuals were assigned to the Mediterranean RMU, except for the two individuals from the hybrid nest from 2017 (SP02), which showed mixed assignment probabilities. In previous studies, a higher proportion of Atlantic-origin individuals in Spanish nests was reported (Carreras et al., 2018) where 6 out of the 9 nests laid in the Spanish coast with genetic data were determined to be from Atlantic origin and another one was labeled as a potential hybrid. These results agreed with the studies of the origin of individuals in nearby areas (Carreras et al., 2006; Clusa et al., 2014) and supported the idea that these new nesting events were related to juveniles in the area that mature and laid a nest before returning to their populations of origin. However, our results show an almost residual presence of Atlantic individuals both in the nests laid in the Spanish coasts (present study) but also in the composition of juveniles in nearby foraging areas (Chapter 3.1). Several non-exclusive hypotheses can explain these differences. Firstly, these discrepancies could be caused by the differences in the methodology, as earlier studies relied on a few nuclear microsatellites and mitochondrial DNA and the baseline did not include all potential RMUs of origin. In

this chapter, we utilized genome wide thousands of nuclear markers for origin Individual Assignments, providing more reliable results. However, the striking differences suggest that other causes can be causing temporal differences in the relative abundance of Atlantic individuals in the Western Mediterranean, including the recent increase of the Mediterranean populations due to conservation efforts (Mazaris et al., 2017). Or even changes in the Atlantic Meridional Overturning Circulation (AMOC), the current system carrying turtles from the Atlantic nesting populations into the Mediterranean (Ditlevsen & Ditlevsen, 2023). Temporal studies on both nesting and foraging areas using the same methodology are advised to assess the extent of temporal fluctuations in the origin of the turtles.

The analysis of the origin at SubRMU level also raised interesting questions. The seven Mediterranean nests corresponded to six nesting females, as nests SP07 and SP11 were laid by the same female (Chapter 3.2). The finding of two related nests being originated in Greece and the remaining five being admixed contrast with the results presented in Chapter 3.1, where juvenile individuals from the Catalan Coast (<59cm Curved Carapace Length=CCL) in the foraging areas are mostly assigned to the Levantine SubRMU, some to the Greek SubRMU and with a very low presence of admixed individuals. Admixed individuals from different SubRMUs are found in the Mediterranean nesting populations (Barbanti et al., -, in prep) and foraging areas (Chapter 3.1) but its presence is generally low, suggesting that the mating opportunities among individuals of different SubRMUs may not be abundant. Thus, the high presence of admixed nests suggests that the nesting activity in the Western Mediterranean may increase the chances of mating of two individuals from different SubRMUs that mature before returning to their natal beaches, in a similar way that it opened the possibility of mating between individuals from different RMUs. Another reason for the differences between the composition of the nesting and foraging areas in the Western Mediterranean could be related to the sampling effort. For instance, only foraging individuals from the Catalan coast have been analyzed along the Spanish coast (Chapter 3.3),

while the composition of other nearby foraging areas may be different depending on the prevailing currents (Carreras et al., 2006; Clusa et al., 2014). Future studies sampling the remaining foraging areas in the Spanish coast are desirable to fully understand the relationship between the foraging juveniles and the nesting individuals. Finally, the timing of arrival and return to the natal beaches can be different among RMUs and among SubRMUs, leading to ontogenic differences in the composition of foraging turtles. Individual Assignments on subadult and adult foraging turtles in the nearby areas would help to assess if there are potentially ontogenic differences in the composition.

It is worth mentioning that for some nests, different results were obtained for different individuals. This is especially evident in the nest SP09, with two individuals of Levantine origin and the remaining hatchlings behind admixed between Levantine and Greece but can also be seen in other nests. Different assignation results within a nest can be indicative of multiple paternity, a phenomenon that is not rare in the loggerhead sea turtles (Zbinden et al., 2007) and has also been reported in the Western Mediterranean emerging nesting populations (Carreras et al., 2018). This highlights the importance of sampling several individuals per nest to be able to perform fine scale analysis to evaluate multiple paternity but also the origin of the colonizing individuals. For instance, the nest SP09 was likely laid by a female of Levantine origin who had mate with at least two males, one from Greece and another one from the Levantine.

This study demonstrated the potential of genomic based Individual Assignments to the study of the emerging nesting populations in the Western Mediterranean. However, sampling size was necessarily low, due to the nature of the colonization. Consequently, it would be valuable to increase the number of samples and nests over time to make an accurate assessment of the origin of this emerging loggerhead sea turtle population in Spain. It is advisable to use the same genomic methodology and integrate samples from nesting events prior to those presented here, as well as to continue sampling each new season to evaluate whether there is temporal variation in the origin of breeding

individuals or whether the origin assignment of the first nests was biased due to the low resolution and number of nuclear markers.

As a summary, this study showcased the potential of genomics to determine the origin of these nesting events and to detect hybridization among RMUs and admixture among SubRMUs offering the opportunity to establish a robust methodology to track how the species is increasing the range of nesting areas and adapting to a rapidly changing environment.

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04 GENERAL DISCUSSION

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4. GENERAL DISCUSSION

Biodiversity is currently facing a mass extinction, with significant risks to ecosystems and species due to global change and biodiversity loss (Román-Palacios & Wiens, 2020). It is crucial to understand the challenges facing wildlife populations and apply available conservation tools. Important topics in wildlife conservation include identifying conservation units, assessing population size and connectivity, detecting hybridization, and evaluating the capacity of populations to persist and adapt to environmental changes, which is essential for informed conservation management.

Genomics has emerged as an important tool in conservation strategies and, within this "genomic era", single nucleotide polymorphisms (SNPs) have become the preferred molecular marker to study species at population level and allow for better monitoring and conservation of vulnerable populations. One of the features of this technique is that it allows for a reduced representation of the genome and has demonstrated a high capacity to quantify hundreds of thousands of loci in large populations, making it suitable for population genomics research (Carreras et al., 2020; Torrado et al., 2020).

Sea turtle species, recognized for their longevity, fertility, physiological adaptability and migratory abilities, are often considered resilient species (Novelletto et al., 2016). However, they are facing habitat change, loss and degradation at an unprecedented rate. Coastal development, pollution, climate change and other human activities increasingly threaten sea turtles in their nesting and foraging areas (Witt et al., 2010). There is therefore a need to obtain as much information as possible to assess how turtles are coping with the challenge. The present thesis has contributed to answer some of these issues, building on the knowledge from the population genomics of the

loggerhead sea turtle *Caretta caretta* in three Regional Management Units (RMU) to evaluate two major stages of its life cycle, juvenile (foraging areas) and nesting stages (new emerging populations). We have designed different baseline dataset to unravel the stock composition of individuals in four foraging areas in the Mediterranean Sea by successfully performing individual assignments of the sampled turtles and the origin of breeders of sporadic nesting in the Western Mediterranean. The resulting research has contributed to the understanding of the species distributed throughout the Mediterranean Sea, while confirming the power of using reduced representation genomic techniques in species with large genomes.

A genomic baseline to assign them all: The importance of the tool

The loggerhead sea turtle (*Caretta caretta*) is considered a non-model species due to its highly migratory behavior, which often involves complex life cycles that complicate both population studies and conservation efforts. In this thesis, we employed advanced genomic techniques to examine the genetic structure of nesting populations in the Atlantic and Mediterranean, using this data as a baseline to assign individuals from Mediterranean foraging grounds to their populations of origin. This approach not only provides new insights into the species; biology but also offers a replicable methodology for linking breeding populations with individuals in distant areas, thereby contributing to broader conservation efforts.

Intermediate levels of genetic structuring between species and populations are often overlooked in population genetic studies, but they are crucial for understanding fine-scale structuring (Carreras et al., 2020). In marine turtles, the concept of Regional Management Units (RMUs) was proposed to address the need for conservation units that encompass common aggregation areas used by distinct populations (Wallace et al., 2023; Wallace et al., 2010). However, while genetic data identified differentiated nesting populations, RMUs were mainly supported by non-genetic data, such as satellite

telemetry (Shamblin et al., 2014). Using a large set of SNP markers, this study is the first to fully support the genetic structure of three RMUs in the Atlanto-Mediterranean region based purely on genomic data, revealing a real intermediate level of genetic differentiation in the species.

The degree of differentiation among the three RMUs reveals that the Mediterranean RMU is the most genetically distinct from the two Atlantic RMUs. The species phylogeography has unresolved aspects, such as how the loggerhead sea turtle colonized different regions globally. Some suggest that the Mediterranean was colonized by individuals from the Western Atlantic (Shamblin et al., 2014), while others propose a route from the Indian Ocean via South Africa, followed by migration to the Atlantic RMUs (Baltazar-Soares et al., 2020). These hypotheses, however, are based solely on mitochondrial data, which can conflict with nuclear genome results (Galià-Camps et al., 2024). Future genomic studies covering more populations may help resolve these questions, as has been shown in species with complex evolutionary trajectories (Galià-Camps et al., 2024).

The Regional Management Units (RMUs) are not the only intermediate level of genetic structuring above the population level in loggerhead sea turtles. A reanalysis of genomic data confirmed significant genetic structure within the Mediterranean RMU, suggesting multiple hierarchical layers (Barbanti et al., -, in prep). Early efforts to define the species genetic structure relied on mtDNA and microsatellites, aiming to identify distinct Management Units (MUs) for conservation (Moritz, 1994). Initial studies described four MUs in the Mediterranean, later increasing to five (Clusa et al., 2018) and seven (Shamblin et al., 2014) based on different sample sites and different number of markers used, demonstrating that more markers improve the detection of genetic differentiation (Clusa et al., 2018). However, recent genomic data revealed deeper structuring, with almost all populations showing significant differences, except for some in the Levantine region (Cyprus, Turkey) and mainland Greece (Barbanti et al., -, in prep). Notably, genetic analysis grouped these populations into three main clusters: Sirte (Lybia),

Levantine (including ISR, LEB, ALA, AKA, BEL, DAL), and Greece (MES, RET, KYP, ZAK), a pattern supported by particle modeling of hatchling dispersal (Casale, 2015). This clustering aligns with the idea of "Subregional Management Units" (SubRMUs), an additional hierarchical level of conservation proposed to reflect similar dispersal trajectories (Casale & Mariani, 2014). Thus, the loggerhead sea turtle exhibits a confirmed hierarchical genetic structure at three levels—populations, SubRMUs, and RMUs, which is crucial for building accurate baselines to assess turtle origins at sea.

In this thesis, we built a genomic baseline using data from three Regional Management Units (RMUs) through the same reduced representation genomic technique (2bRAD sequencing). During the analysis, different processes were proceeded to follow the better genotyping strategy. Genotyping unknown samples simultaneously with baseline samples recovered all polymorphic markers, while independent genotyping resulted in the loss of markers that were polymorphic in the baseline but absent in the unknown samples. The use of BCFtools retains only polymorphic positions relative to the reference genome, meaning the number of loci recovered depends on the number and genetic diversity of the samples genotyped (Li, 2011). Independent genotyping, especially with a low number of unknown individuals, can significantly reduce marker recovery and compromise assignment accuracy. Thus, joint genotyping is recommended for future studies to maximize marker recovery and genetic variability, improving assignment probabilities.

A key issue when building baselines, is whether uneven sample sizes across genetic clusters in the baseline a bias population structure analyses and individual assignments. This concern was particularly relevant in our work at the RMU level, where the Mediterranean RMU was overrepresented compared to the two Atlantic RMUs. Using the full dataset (RMU Baseline) versus a reduced dataset (RMU Baseline-R) produced different outcomes depending on the analysis. While two of the genetic structure analyses showed minimal differences between datasets, the third with the complete dataset

struggled to distinguish the two Atlantic RMUs effectively, likely due to known issues with uneven sample sizes in the analyses (as observed in the Chapter 3.2). The differences between clusters, despite using the same data, reflect their distinct approaches.

During the assignment analysis of individual assignments from foraging grounds using both RMU baseline datasets, a significant observation was that all individuals were consistently assigned to the same RMU across tests. This consistency indicates that the genetic variability within the baseline is adequate for differentiation at the Atlantic-Mediterranean level, regardless of sample sizes. However, individuals from foraging grounds assigned to Atlantic RMUs showed lower assignment probabilities when using the complete dataset. Although both analyses were robust, addressing sample size disparities among potential origins in the baseline is crucial for enhancing the assignment of underrepresented groups. Furthermore, individuals from foraging grounds were assigned to the Mediterranean (MED) and Northeast Atlantic (NEA) RMUs with higher probabilities than to the Northwest Atlantic (NWA) RMU. This disparity may stem from the limited representation of nesting populations in the NWA RMU, which may not adequately reflect its genetic variability. Therefore, there is an opportunity to enhance sampling efforts in the NWA RMU, particularly along the Florida coast, which hosts one of the largest loggerhead turtle aggregations globally, as well as in important nesting areas in the Caribbean Sea. Increasing the genetic variation included in the Atlantic baseline representation would improve the resolution of genetic assignments.

The power of all baseline datasets was confirmed by high self-assignment accuracy and membership probability analyses. Selecting the appropriate model is crucial, with the SVM model consistently demonstrating high assignment accuracy by Monte Carlo cross-validation across all scenarios. The SVM model outperformed others, likely due to its ability to analyze high-variance datasets, maximize separation margins between classes, and its robustness against outliers, allowing for specific group-level variability considerations (Ripley et al., 2016).

Reliable individual assignments at the RMU level were performed for all samples and at the SubRMU level for individuals assigned to the Mediterranean RMU. Individual assignments (IA) outperformed microsatellite analysis (MSA) in assessing individuals' origins in foraging areas, as IA operates at the individual level without large confidence intervals (Carreras et al., 2011). The genomic approach carried out in this thesis represents a significant advancement, identifying individuals from all three RMUs in the baseline, while previous mtDNA and microsatellite studies considered only two RMUs due to the absence of NEA baseline samples. Additionally, previous studies reported 13% to 22% of individuals not assigned to any RMU due to resolution or amplification issues, with some foraging areas reaching 35% (Clusa et al., 2016; Piovano et al., 2011). In contrast, our genomic approach successfully assigned all 103 samples from foraging individuals to a single RMU with high probabilities, attributing this success to the increased number of markers and an extended baseline. For the first time, a hierarchical approach enabled the determination of origins from specific SubRMUs within the Mediterranean RMU, although 13 individuals showed admixed assignments when analyzing three SubRMUs, compared to four individuals with two SubRMUs. The number of genetic clusters used in the assignPOP tool influenced assignment results, as it tends to favor assignments to the closest genetic cluster. The presence of admixed individuals, particularly those showing intermediate probabilities for Libya and the Levantine region, indicates that these assignments likely reflect true admixture rather than methodological resolution issues (Barbanti et al., -, in prep). This study is the first to identify origins at a subregional level within the Mediterranean and document admixture with unprecedented precision for individuals found in foraging areas.

The genomic-based individual assignment analysis revealed that loggerhead sea turtles from various origins (NWA, NEA, and MED) aggregate in juvenile stages across different Mediterranean foraging areas, highlighting a significant heterogeneity in origin composition among these locations. This finding aligns with earlier mitochondrial multilocus studies, which suggested that the observed heterogeneity may be influenced by

prevailing ocean currents (Carreras et al., 2006; Clusa et al., 2014). Interestingly, the proportion of Atlantic individuals in the Catalan Coast and Lampedusa was significantly lower than previous estimates, which reported 20-30% and up to 90% respectively. This discrepancy may reflect changes in foraging ground composition over time and requires caution when comparing results due to variations in methodology and confidence intervals. Potential explanations for the observed decline in Atlantic individuals include a recovery of Mediterranean nesting populations due to conservation efforts (Mazaris et al., 2017) and alterations in the Atlantic Meridional Overturning Circulation (AMOC), which may affect turtle distribution patterns (Ditlevsen & Ditlevsen, 2023). Future studies employing consistent methodologies over time are necessary to evaluate shifts in foraging ground composition, particularly in the context of global warming.

The majority of individuals sampled in the Mediterranean foraging areas originated from the Mediterranean Regional Management Unit (RMU), and the analysis at the SubRMU level revealed distinct differences in individual composition among various foraging grounds. This study marks the first reporting of individual assignments at the SubRMU level, building upon previous insights from mitochondrial DNA multi-locus analyses (MSA) that focused solely on the Catalan coast (Clusa et al., 2014). A notable difference in our findings was the reduced percentage of individuals from Libya in the Catalan coast, which contrasted with prior estimates that indicated approximately 39% from this region, highlighting the variability in sampling results. Additionally, the MSA is known to produce high confidence intervals due to the presence of common haplotypes, which can obscure the true genetic diversity present (standard deviation of 29%). Our results also support the hypothesis that migration from nesting areas to foraging grounds is driven by the main current system (Millot & Taupier-Letage, 2004) and align with predictions of hatchling dispersal derived from particle modeling (Casale & Mariani, 2014). Furthermore, we observed that the western Aegean coast had a higher contribution from Greece, while the eastern coast was more influenced by individuals from the Levantine SubRMU, indicating significant compositional differences within the Aegean

Sea. The unexpected dominance of Levantine individuals in the Catalan coast, along with a reduced representation from Greece, may be attributed to the recent expansion of Turkish populations in the Mediterranean (Casale et al., 2018), warranting further investigation. Future studies applying similar methodologies across additional foraging areas are essential to gain a more comprehensive understanding of juvenile migration routes in the Mediterranean.

In this thesis, we propose a comprehensive methodology to study the origin of turtles in foraging ground aggregations, adaptable to other migratory species. We provide a genomic baseline for assigning individuals of unknown origin at various levels, along with a detailed list of markers used in the analysis. The use of these baselines can be summarized in the following steps (Figure 1):

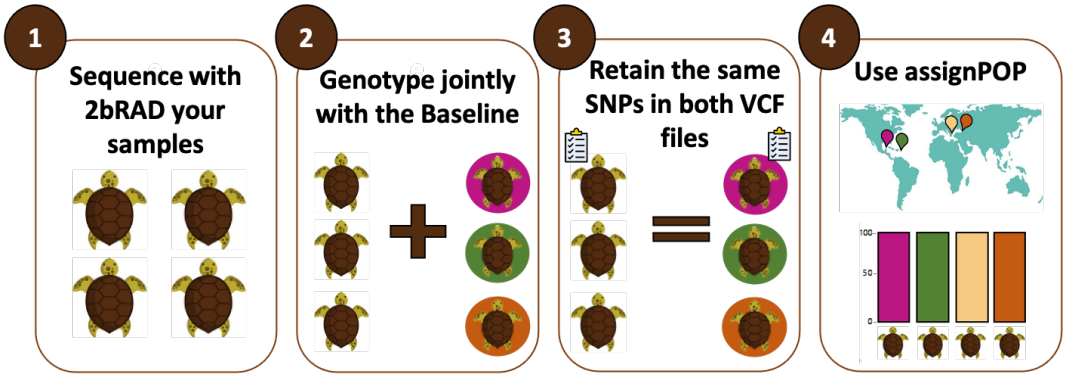

Figure 1: Baseline User Guide. Steps to perform an Individual Assignment of *C. caretta* at RMU and Sub RMU level: 1. Sequence with 2bRAD your samples of interest using the methodology described. 2. Genotype jointly with the Baseline. Given the results obtained in the Chapter 3.1, it is recommended that once mapped against the species genome, the samples of interest be genotyped at the same time as the samples belonging to the RMU baseline. 3. Retain in the unknown samples VCF the same SNPs as in the selected baseline dataset. Within the framework of this thesis, we have provided the SNPs list of each baseline (File S1, File S2 and File S3). 4. Use assignPOP to estimate the percentage of assignment of the samples of interest using the SVM model in comparison to the selected baseline.

PopCorn' nesting in the Western Mediterranean: The conquest of *Caretta caretta*

The loggerhead sea turtle is well-documented as the most abundant sea turtle species in the Mediterranean Sea, with its historical nesting sites primarily located in the Eastern Mediterranean. In recent years, however, an increase in the number of loggerhead sea turtles (*Caretta caretta*) nests has been observed on the Western Mediterranean linked to climate change and the increase in the temperature of the Mediterranean Sea (Cardona et al., 2023; Hochscheid et al., 2022). This shift has prompted researchers to investigate not only the causes behind this change but also the origins of the turtles now nesting in these regions.

One of the hypotheses is that warmer sea surface temperatures make easier for female loggerhead turtles to lay eggs further north, which has led to the species nesting in new areas such as the Mediterranean coasts of Spain, where previously this type of nesting events were rare. This trend has been observed in recent years, with more nests appearing each year, especially in regions such as Catalonia and Valencia.

Rising temperatures can influence the reproductive behaviour of turtles, altering the timing of nesting seasons and the geographic locations where females find suitable conditions for nesting. Furthermore, climate change affects the thermal conditions of the sand where eggs are incubated, influencing the sex ratio of hatchlings, as warmer sand temperatures tend to produce more female hatchlings (Booth, 2017) In this way, we have proposed a colonization model that proposes that females born at these emerging sites will grow and, upon reaching reproductive maturity, will return by philopatry to the beaches where they were born (Chapter 3.2). Therefore, this thesis proposes that we are witnessing the emergence of a new nesting area in the Mediterranean Sea, if environmental conditions remain suitable and facilitate hatching success.

However, this change also presents challenges, as these beaches may not yet have the protection measures or management strategies that have been developed at traditional nesting sites. Ongoing monitoring and conservation activities are crucial to safeguard these emerging nesting sites.

This study found that most nests produced viable hatchlings, though hatching success varied widely (0 to 93.1%) across nests, a pattern noted in previous research in the Western Mediterranean (Carreras et al., 2018; Hochscheid et al., 2022) This variability could stem from various factors, such as local environmental conditions, management strategies, and the biological conditions of the parents. Recent analyses indicate that the window for viable nesting has expanded in the last decade due to rising temperatures (Cardona et al., 2023), which may increase female hatchling proportions (Carreras et al., 2018; Santidrián Tomillo et al., 2023). This high female offspring percentage could lead to more individuals returning as breeding adults, thus reinforcing the new population establishment.

Mitochondrial DNA sequencing revealed specific haplotypes associated with Atlantic and Mediterranean regions, including one (CC-A31.1) linked to eastern Mediterranean populations (Clusa et al., 2013; Garofalo et al., 2009), and another (CC-A1.1) from the Atlantic (Shamblin et al., 2014). This mixed origin suggests potential genetic admixture, which could enhance offspring fitness through hybrid vigor, though it might also risk outbreeding depression (Edmands, 2007; Fitzpatrick et al., 2020). This is where the necessity of establishing genomic baselines is evident for understanding this emerging population genetic viability (Shamblin et al., 2014).

The recent surge in nesting activity in the Western Mediterranean suggests the beginning of a new loggerhead population (Hochscheid et al., 2022). The establishment of this population will likely accelerate once females return to nest, driven by philopatry

(Carreras et al., 2018). However, up to now genetic analysis using SNP markers showed that individuals from different nests from different years were not laid by the same female, and this rules out remigrant females. However, we identified two nests laid by the same female within the same nesting season.

Sea turtles of the species *Caretta caretta* exhibit a reproductive strategy that involves laying multiple nests during a single nesting season. This behavior, known as multiple clutching, is intended to increase reproductive success in environments characterized by high hatchling mortality. Each female may lay nests at different times of the season, typically with intervals of a few weeks between nesting events (Miller, 2017). Additionally, this strategy allows females to distribute their nests across various locations, thereby mitigating the risks by predation or unfavorable environmental conditions. We could identify this behavior in two nests laid by the same re-nesting female, the same year and 213 km apart.

Monitoring rising temperatures effects on sex ratios, fitness, and hatchling survival is crucial (Dimitriadis et al., 2018). Since emerging nests occur on anthropized beaches, human impacts like light pollution and coastal erosion must be studied. Applying scientifically informed monitoring into active conservation strategies can help ensure the species' growth and survival as global temperatures continue to rise. We are documenting the early stages of this colonization process, providing a unique opportunity to study this adaptive process from the beginning

The colonization of the Western Mediterranean by loggerhead turtles exemplifies adaptation to climate change. The application of genomic tools, developed in the first part of this thesis identified distant Regional Management Units (RMUs) as the origin of these colonizers, but the use of mtDNA and microsatellites limited precise assignments and obscured potential hybridization processes. The genomic techniques presented

in Chapter 3.1 were applied to individual turtles from emerging Spanish nests to trace their origins and better understand potential genetic admixture and its implications for population viability.

We considered to assessing in a hierarchical way the origin of the significant individuals as described in Chapter 3.1, to assign them first at the RMU level using the RMU Baseline-R, then within the MED RMU we used the MED Baseline, and in an integrative analysis, we decided to evaluate the individual assignment considering five genetic clusters by using the RMU Baseline. The genomic method allowed successfully determined the origin of all eight nests, assigning them not only to broader RMUs but also to specific sub-regions.

Globally, we found hybrid individuals, such as those from the one nest (SP02), which presented an Atlantic mtDNA haplotype but resulted in being a hybrid between Atlantic and Mediterranean RMUs. Hybridization between these regions has been observed before (Carreras et al., 2018), and although previous studies demonstrated genomic isolation (Carreras et al., 2011), the ongoing colonization may increase admixture. Moreover, combining information of the maternal inherited (Chapter 3.2) and the genomic IA, it is possible to identify the breeders of a nest. In this case, we can infer that NWA female reproduced with a male from Lybia, being the breeders of nest SP02. Hybridization between different RMUs could be at risk if involving outbreeding depression, potentially reducing fitness (Frankham et al., 2010). The viability of hybrids is unknown, as data on hatching success was unavailable, but some nests with low success might indicate potential negative effects (Hochscheid et al., 2022).

Nearly all individuals from the 2016-2019 Spanish nests were assigned to the Mediterranean RMU, except for two from the hybrid nest, which exhibited mixed assignment probabilities. Previous research indicated a higher presence of Atlantic-origin individuals

in Spanish nests (Carreras et al., 2018). Our findings suggest a minimal presence of Atlantic individuals in the nests and nearby juvenile foraging areas. This discrepancy may arise from differences in methodology, with our genome-wide approach offering more reliable results. Other factors could also contribute, including the rise of Mediterranean populations due to conservation efforts (Mazaris et al., 2017) and changes in the Atlantic Meridional Overturning Circulation (Ditlevsen & Ditlevsen, 2023).

The analysis at the SubRMU level revealed that the seven Mediterranean nests corresponded to six nesting females, with two nests laid by the same individual (Chapter 3.2). Interestingly, the two related nests had high probability of being from Greece meaning that both breeders, female and male, originated from Greece. The remaining five nests exhibited admixture between the Levantine and Greece SubRMU. This contrasts with the origin of juvenile turtles foraging the Catalan Coast, which primarily belonged to the Levantine SubRMU, with a low presence of admixed individuals. The increased admixture in Spanish nests suggests that Western Mediterranean nesting could enhance mating opportunities between individuals from different SubRMUs. Future research should sample additional foraging areas along the Spanish coast to better understand the relation between foraging juveniles and nesting individuals. Finally, variations in the timing of arrivals and returns to natal beaches among RMUs could lead to ontogenic differences in foraging turtle composition. Assessing individual assignments in subadult and adult foraging turtles may provide insight into these potential differences.

The assignment probabilities could show variability between hatchlings from the same nest. For instance, one nest contained two individuals of Levantine origin while others showed admixture between Levantine and Greek origins. Such mixed results within the same nest could suggest multiple paternity, a phenomenon not uncommon in loggerhead sea turtles (Zbinden et al., 2007) and has been observed in emerging populations in the Western Mediterranean (Carreras et al., 2018). This underlines the necessity of sampling multiple individuals per nest to facilitate detailed analyses of both paternity

and the origins of colonizing individuals.

This study demonstrated the effectiveness of genomic-based Individual Assignments in analyzing emerging loggerhead sea turtle nesting populations in the Western Mediterranean, despite the inherently low sampling size due to colonization dynamics. Increasing the number of samples and nests over time is essential for accurately assessing the population origins. Utilizing consistent genomic methodologies and integrating samples from earlier nesting events will help identify potential temporal variations in breeding origins and address any biases from previous studies. Overall, this research showcases genomics; potential to track hybridization and adaptation in response to environmental changes.

This thesis makes significant contributions to the conservation of non-model species, such as the loggerhead sea turtle (*Caretta caretta*), through the application of advanced genomic tools that reveal the genetic structure of populations at different stages of their life cycle. The findings provide crucial insights for sea turtle conservation and establish a replicable methodology for other highly migratory species. By constructing a robust genomic baseline for individual assignments across the Mediterranean and Atlantic, this research successfully connects breeding populations to individuals in distant foraging grounds, uncovering a more complex genetic structure than previously understood. The study emphasizes the importance of genomics in identifying regional and subregional management units, enabling more informed and effective conservation strategies. Furthermore, it offers a means to monitor population shifts potentially driven by global warming and habitat alteration, underscoring the ongoing need for targeted conservation efforts. The methods and insights developed here are essential not only for loggerhead turtles but also for the broader conservation of other migratory species facing similar environmental challenges.

05 CONCLUSIONS

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5. CONCLUSIONS

1. The loggerhead sea turtle presents a clear hierarchical genetic structure in the studied area, presenting three levels of differentiation all of them supported by genomic data: the Regional Management Units (RMUs), subRegional Management units within the Mediterranean and at the Management Unit (population) level.

2. We have developed a methodology to perform genomic Individual Assignments that is powerful enough to assess the origin of foraging individuals) at RMU or SubRMU level.

3. The composition of the juveniles in Mediterranean foraging grounds is not homogeneous, with a general prevalence of individuals from Mediterranean origin and more specifically from the Levantine region.

4. The origin of the individuals across foraging areas and with previous studies, show temporal and geographic differences.

5. The emerging nests on the Mediterranean coast of Spain are laid by new colonizing individuals rather than remigrants, although a renesting female within the same season was detected. Thus, the increase of nesting events seems linked to the arrival of new colonizers

6. Genetic data, both mitochondrial but particularly genomic data, confirmed that the colonization of the western Mediterranean as a new nesting area for the loggerhead turtle can originate from individuals from the Atlantic and Mediterranean RMUs with potential risk of outbreeding depression in the context of the colonization.

7. A high percentage of nests resulted from the admixture of individuals from different SubRMUs within the Mediterranean, thus suggesting that the new area may facilitate admixture process that are rare in regular nesting areas.

8. As a global final conclusion, genomic approaches based on thousands of genome-wide markers are powerful tools to provide novel insights in species with complex life cycles, such as the loggerhead sea turtle.

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IN MEMORY OF THE PEOPLE I LOVE WHO EMBARKED ON THEIR OWN JOURNEY WHILE I WAS DIVING WITH TURTLES

JUAN LUIS FLORES COLIN
TRINIDAD RUIZ OLIVERA
GABRIEL AVENDAÑO RAMOS