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Incipient Range Expansion of Green Turtles in the Mediterranean

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ABSTRACT

In response to global climate change, numerous taxa are expanding their living ranges. In highly migratory species such as sea turtles, this expansion may be driven by individuals from nearby or distant areas. Recent nests outside the species' typical nesting range and reports of adult-sized individuals in the western Mediterranean suggest a green turtle (*Chelonia mydas*) range expansion into the central and western Mediterranean. To assess the green turtles' origin in these novel habitats, we built a genomic baseline using 2bRAD sequencing on five individuals from each of three Regional Management Units (RMUs): North Atlantic, South Atlantic and Mediterranean. We then compared this baseline with genotyped hatchlings from three nests laid in new central and eastern Mediterranean sites and four mature-sized green turtles tagged with satellite telemetry in the western Mediterranean. Our analyses revealed that the Tunisia nest originated from the South Atlantic RMU, while the Crete nests were produced by turtles from the Mediterranean RMU. Additionally, the three adult-sized turtles sampled in the southwestern Mediterranean were assigned to the South Atlantic RMU, while the mature-sized individual sampled in the northwestern Mediterranean belonged to the Mediterranean RMU. These results suggest a simultaneous incipient colonisation by two geographically distant RMUs. We propose that the range expansion of green turtles into the central and western Mediterranean is likely climate driven and these populations may become

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globally important as temperatures rise. Finally, our results highlight the essential role of the cost-effective RAD-Seq genomic assessment combined with tagging data to understand potential new colonisations.

1 | Introduction

The Mediterranean Sea is one of the most ecologically vulnerable regions on the planet as it is both a hotspot of global change (García-Ruiz et al. 2011; Underwood et al. 2009) and biodiversity (Myers et al. 2000). In response to global warming, marine animals may alter their distributions, which can influence species interactions and the functioning of marine ecosystems (Parmesan et al. 2003; Poloczanska et al. 2013; Pörtner et al. 2014).

Global climate change has acute effects on animals whose life-history traits, behaviour and physiology are directly tied to ambient temperature, such as sea turtles (Fuentes et al. 2010; Patrício et al. 2021). Sea turtle eggs develop within a narrow thermal range (23°C–35°C, Ackerman 2017) and their sex determination is temperature-dependent (Mrosovsky 1994; Yntema and Mrosovsky 1980). Additionally, the increased sand temperatures related to climate change can negatively influence hatching success (Matsuzawa et al. 2002; Santidrián Tomillo et al. 2015) and posthatching fitness (Booth et al. 2004). Moreover, sea level rise limits suitable nesting sites due to flooding and coastal erosion (Dimitriadis et al. 2022; Feagin et al. 2005; Fuentes et al. 2010; Poulter et al. 2009). Although sea turtles could mitigate these impacts by finding new suitable nesting sites (i.e., Pike 2013), their nest site fidelity (i.e., Meylan et al. 1990) is thought to limit dispersal to new nesting areas. In addition, altered sea currents (Böning et al. 2008; Franco et al. 2020) significantly affect turtle movements (Hays 2017), influencing nesting locations and hatchling dispersal (Boyle et al. 2009; Putman et al. 2010) and potentially hindering their dispersal capabilities (Lohmann et al. 2008). These combined climatic stressors are considered a significant global challenge to sea turtles' resilience.

Likely in response to climate change, loggerhead turtles (*Caretta caretta*) are colonising new nesting beaches in the western Mediterranean basin (Cardona et al. 2023; Carreras et al. 2018; Hochscheid et al. 2022; Luna-Ortiz et al. 2024 and references therein), despite the potential limitations in sea turtles mentioned above. Yet only a very few records of migration to new nesting sites have been observed for the green turtle (*Chelonia mydas*), the second most abundant species in the Mediterranean (Casale et al. 2018 and references therein). Green turtle nesting sites in the Mediterranean are currently concentrated in the eastern Mediterranean in Turkey, Cyprus and Syria (Kasparek et al. 2001; Rees et al. 2008), with minor nesting habitats in Israel, Lebanon and Egypt (Kasparek et al. 2001). However, climate change rising temperatures in the Mediterranean (Pike 2013) are decreasing the suitability of these areas and producing population feminisation (Önder and Candan 2016). At the same time, they are potentially increasing the nesting suitability of the central basin for green turtles in the near future (Arslan et al. 2023; Mancino et al. 2023). In the past two decades, isolated green turtle nesting events have been detected in regions out of the regular nesting range, namely in Tunisia (Ben Ismail et al. 2022), Greece (Margaritoulis et al. 2019, 2023) and Libya (Saied et al. 2023). Green turtles primarily inhabit the eastern Mediterranean, covering the Levantine basin (Casale et al. 2018), Albania (Piroli and Haxhiu 2020), Greece (Margaritoulis and Teneketzis 2003) and North Africa (Broderick et al. 2007). Historically, their presence in the western Mediterranean has been considered sporadic (Bentivegna 2002; Camiñas 2002), typically limited to less than five juvenile sightings per region (Italy, France, Spain, Morocco and Tunisia) (Bentivegna et al. 2011; Carreras et al. 2014; Casale and Margaritoulis 2010). In addition to these nesting events, mature-size individuals have been recently detected for the first time in the western basin. Consequently, the green turtle may be in the initial stages of expanding its distribution by starting to potentially colonise new nesting areas, as has been observed in loggerhead turtles in the western Mediterranean (Hochscheid et al. 2022).

In this context, studying this potential colonisation is crucial to determine the origin and routes of these putative colonisers nesting in new Mediterranean areas. For sea turtles, Mixed Stock Analyses (MSA) based on mtDNA have been used to estimate the origin of individuals found in foraging grounds (i.e., Tikochinski et al. 2018). However, this methodology has several limitations, including the inability to assign individuals due to common haplotypes (i.e., Shamblin et al. 2018), the need for large sample sizes to obtain reliable estimates (Patrício et al. 2017), and wide confidence intervals (Barbanti et al. 2019). Individual assignments using nuclear and mitochondrial markers have also been developed for the loggerhead sea turtle (Carreras et al. 2011; Revelles et al. 2007) to trace the origin of turtles in foraging areas (Clusa et al. 2016; Piovano et al. 2011) or to infer the origin of colonisers of the western Mediterranean nesting events (Carreras et al. 2018). However, all these analyses rely on the genotyping of the potential nesting regions of origin (baseline) and need large numbers of genetic markers to be powerful enough to discriminate among populations (Clusa et al. 2018).

Despite being the first sea turtle species with a reference genome (NCBI RefSeq assembly: GCF_015237465.2), the potential of genomics to assess population structure and origin assignment of green turtles at the Mediterranean–Atlantic regional level remains untested. Genetic methods, which are instrumental in defining Regional Management Units (RMUs), provide a reliable approach for enhancing studies involving these animals (Barbanti et al. 2019, 2022). RMUs integrate multiple data sources—nesting sites, population abundances and trends, population genetics and satellite telemetry—to define groups of populations that share migration routes and foraging areas, thereby facing similar threats (Wallace et al. 2023, 2010). Accurate individual assignments require a genomic baseline with individuals from the different RMUs (Wallace et al. 2010) of potential origin. To obtain large numbers of markers, RAD-Seq techniques using base-selective (5′-WN-3′) adaptors offer a cost-effective approach, balancing economic efficiency with reliable genetic differentiation by reducing the genome while preserving genomic signals (Galià-Camps et al. 2022).

In addition to determining the origin of these events, identifying migratory routes is crucial for evaluating distribution ranges, habitat preferences and ecological needs (i.e., Hart and Fujisaki 2010; Seminoff et al. 2008). Satellite tracking complements genetic analysis by revealing green turtle migratory and spatial behaviours (i.e., Godley et al. 2002, 2008; Robinson et al. 2023). Previous studies in the Mediterranean have revealed clear migratory patterns for this species, but only in the eastern basin (Stokes et al. 2015), highlighting the North-African coast as a relevant feeding area for adults (Godley et al. 2002; Rees 2013). Consequently, data from turtles outside these areas can refine migratory routes and habitat use when combined with genetic tools (i.e., Bourjea et al. 2015; Godley et al. 2010). In summary, past research on sea turtles using genetics and telemetry has developed the foundations for studying this recently detected potential colonisation process of green turtles.

In this study, we document several occurrences of green turtles outside the species' typical range in the Mediterranean, identify the origin of putative colonising individuals, and trace their migration routes. To do this, (i) we established a RAD-Seq genomic baseline built with turtles from the three closest and currently established RMUs, analysing their genomic diversity and divergence, (ii) we assigned individuals of unknown origin (hatchlings of sporadic nests and tagged individuals in the western Mediterranean within mature sizes) to their source populations, and (iii) we combined genetics and satellite telemetry information to assess the migratory routes of the tagged individuals. By integrating genomic data and satellite-tracking information, we can start to understand how green turtles can expand their nesting areas and, as a result, propose conservation strategies in the new areas.

2 | Materials and Methods

We combined genetic data with satellite telemetry to investigate the migratory routes of the tagged individuals. This integration of genomic and satellite tracking information allows us to reconstruct their migratory history. Genomic data reveals their origin, while satellite telemetry shows their movement patterns and provides insights into their destinations.

2.1 | Sample Collection

We collected tissue samples from five green turtles at regular nesting beaches within each of the three RMUs (Figure 1a) that could be a potential source of the colonising individuals of unknown origin (breeders of sporadic nests and tagged individuals in the western Mediterranean within mature sizes; Figure 1b). Samples were collected from Mediterranean RMU hatchlings (Tikochinski et al. 2018) (represented by Akamas (AKA) in Cyprus); from nesting females of the North Atlantic RMU (Barbanti et al. 2019) represented by the Cayman Islands (CAY) in the Caribbean; and from nesting females of the South Atlantic RMU (Patrício et al. 2017) (represented by Poilão (POI) in Guinea Bissau) (Table S1). These sites were selected as they represent the closest nesting locations to the central and western Mediterranean for each one of the three considered RMUs. For the Cayman Islands, we used individuals that were likely not related to the local sea turtle reintroduction programme (Barbanti et al. 2019, 2022). In addition, we opportunistically collected samples from newly found nests located outside the nesting area of the species in the Mediterranean, including dead hatchlings found in two nesting events from Crete (Greece) (GN1: one hatchling and GN2: two hatchlings) (Margaritoulis et al. 2019, 2023) and one from Tunisia (GN3: one hatchling) (Ben Ismail et al. 2022) (Figure 1b; Table S2).

Four individuals, one male (GT_1) and three females (GT_2; GT_3 and GT_4), by-caught in the western Mediterranean basin were also sampled for genomics (Figure 1b), satellite tagged (see section Satellite Telemetry for details; Tables S3 and S4) and their reproductive status assessed (see section Reproductive status). However, due to the small size of the sample from GT_1, it

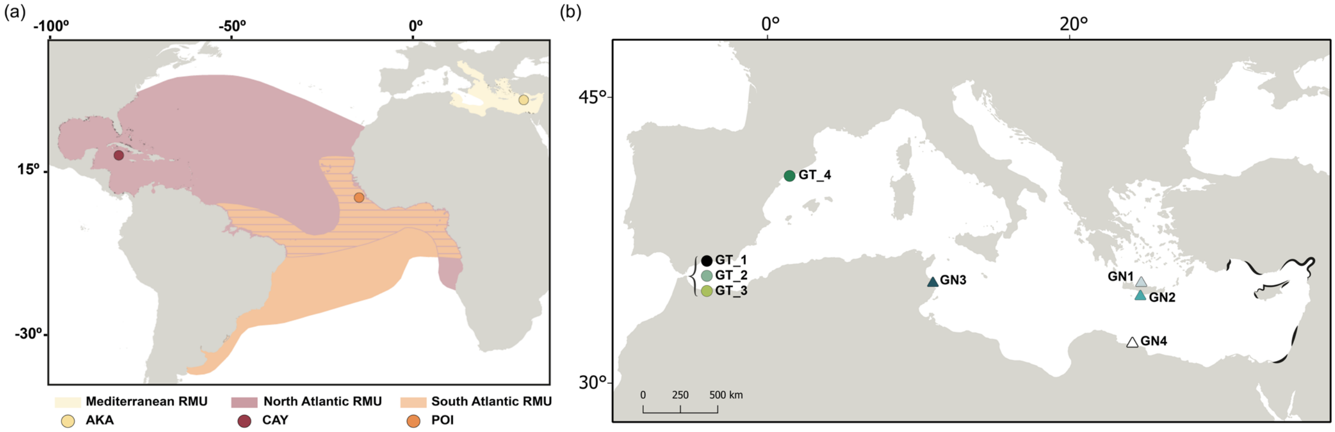

FIGURE 1 | (a) Regional Management Units (Wallace et al. 2023) used for the genetic comparison to generate the genetic baseline. Circles indicate the sampled location within each RMU: Akamas (AKA) for the Mediterranean RMU, Cayman Islands (CAY) for the North Atlantic RMU and Poilão (POI) for the South Atlantic RMU. (b) Sampling locations of the green turtle individuals of unknown genetic origin. Triangles indicate the green turtle sporadic nests (GN) recorded outside of the species' nesting range in the Mediterranean as reported in Table S2. The coloured (i.e., nonwhite) triangles represent those that were analysed using 2bRAD sequencing ($N = 3$ nests; GN1: 1 hatchling, GN2: 2 hatchlings, GN3: 1 hatchling). Circles correspond to the sampling location of adult-sized individuals tagged with satellite transmitters (GT). Green circles were analysed with 2bRAD sequencing ($N = 3$), as coded in Table S1 and the black circle only had D-loop information. The established nesting sites of green turtles in the Mediterranean Sea are marked with a black line. Maps were created using QGIS v. 3.22.9.

was not possible to obtain sufficient genomic DNA for constructing the genomic library for this individual. All muscle and skin samples were preserved in 96% ethanol and stored at −20°C. The collection and transportation of samples were conducted in accordance with national and international legislation.

2.2 | Laboratory Procedures

We extracted DNA using Qiagen Core B blood and tissue kit following the manufacturer's protocol. We quantified DNA concentration and quality using Nanodrop. 2bRAD libraries were obtained for all 22 samples except for GT_1 due to sample failure (Table S1). We performed the 2bRAD genotyping following the optimised 2bRAD protocol for loggerhead turtles (Barbanti et al. 2020) with selective adaptors (Luna-Ortiz et al. 2024). Thus, we constructed individual libraries (Table S1) by digesting 180 ng of DNA (~40 ng/μL) with the AlfI enzyme, and we used base-selective (5′-WN-3′) adaptors. We amplified the ligated DNA with a Taq-high-fidelity PCR, obtaining a final fragment of ~165 bp. We purified the correctly amplified libraries using 90 μL of magnetic beads (SPRIselect), and then we measured with a Quant-iT Picogreen dsDNA Assay Kit (Thermo Fisher Scientific). They were then pooled for 50 bp single read sequencing with a NextSeq2000 sequencer at the Genomics Unit of the Centre of Genomic Regulation (CRG).

As we did not obtain a 2bRAD library for GT_1, we amplified and sequenced ~800 bp of the mtDNA D-Loop control region with the primers LCM15382 (5′-GCTTAACCCTAAAGCATTGG-3′) and H950 (5′-GTCTCGGATTTAGGGGTTT-3′) (Abreu-Grobois et al. 2006). We performed the polymerase chain reaction (PCR) in a final volume of 15 μL containing 2 μL of DNA, 5.08 μL of deionised water, 3 μL of PCR buffer 5× (GoTaq Promega), 1.8 μL of dNTPs (1 mm), 0.6 μL of $MgCl_2$ (25 mm), 1.8 μL of bovine serum albumin, 0.3 μL of each primer (10 μm) and 0.12 μL of GoTaq G2 Flexi DNA Polymerase (Promega, 5 U/μL). Next, we purified 3 μL of the product with 2 μL of ExoSAP (0.4 U of EXO and 0.4 U of TSAP) through a single cycle of incubation at 37°C for 15 min followed by 80°C for another 15 min. Then, we introduced 1 μL (5 μm) of forward primer (LCM15382) and we dried at 80°C for 30 min, preparing the mtDNA amplicons for sequencing on an ABI 3730 automated DNA analyser (Applied Biosystems) at the Scientific and Technical Services of the University of Barcelona.

2.3 | 2bRAD Genotyping and Loci Filtering

We processed the sequences using customised scripts from Barbanti et al. (2020). In brief, we trimmed the raw sequences to 34 bp and mapped them to the green turtle reference genome (GenBank accession GCF_015237465.2) (Bentley et al. 2023) by using Hisat2-2.2.1 (Kim et al. 2019). We performed variant calling of polymorphic nucleotides (SNPs) with BCFtools (Li 2011) using all samples, and obtained a VCF file with the individual genotypes. We used VCFtools version 1.1 (Danecek et al. 2011) to apply quality filters and to select the individuals of the VCF file to obtain different datasets as needed for the different analyses (Table S5). The first dataset included only individuals from established nesting areas, used to generate the genomic baseline (‘Known’ dataset). We removed individual genotypes based on < 5 reads (–minDP = 5) and loci with values of mean depth larger than 100 (–max-meanDP 100). We also only retained polymorphic loci with 2 alleles (–min-alleles 2 –max-alleles 2). We removed all loci found in less than 95% of individuals (–max-missing 0.95) as well as singletons and monomorphic loci by adjusting the minor allele frequency filter (–maf) depending on the number of individuals (maf = 1/2n where *n* is the number of the individuals in the dataset). Next, we filtered the resulting polymorphic loci from the second and third datasets. The second dataset included all sampled individuals outside of the typical range for green turtles in the Mediterranean whose genetic origin is not known (‘Unknown’ dataset). The third dataset included all genotyped individuals (‘Combined’ dataset), comprising both the ‘Known’ and the ‘Unknown’ datasets. Finally, we generated six additional datasets (A—F) by combining all the individuals of the ‘Known’ dataset and, in turn, one individual of unknown origin (GN1_1, GN2_1, GN3_1, GT_2, GT_3, and GT_4), randomly selecting only one individual per nest (Table S5). Since the nest GN2 had two genotyped individuals, we randomly selected GN2_1 for the remaining analyses. In these datasets, we first filtered the individuals and then applied the same initial filters to these individual datasets, in order to obtain a higher genetic variance.

We used the BIOEDIT software (Hall 1999) to align the D-loop sequence of the individual GT_1 with published haplotype sequences compiled in the Archie Carr Centre for Sea Turtle Research (Hall 1999). We identified the potential origin of this individual by considering the presence or absence of its D-loop haplotype in established nesting sites reported in previous studies (Encalada et al. 1996; Formia et al. 2006), as previously done for green turtle individuals found in the Spanish Atlantic and Mediterranean waters (Carreras et al. 2014).

2.4 | Genomic Diversity and Population Structure

We explored the SNPs distribution and quality of the baseline across the genome of the green turtle with the polymorphism found in the ‘Known’ dataset (Table S5). To do so, we used GENALEX version 6.5 (Smouse and Peakall 2012) to conduct a regression analysis between the number of polymorphic loci located in each chromosome and the chromosome size. We also used VCFtools version 1.1 (Danecek et al. 2011) to determine the mean locus depth of all SNPs and represented the mean depth of each SNP along the 28 chromosomes utilising the ‘*ggplot*’ package (Wickham 2006), to check for any potential bias in depth across the genome. We measured genetic diversity and population structure among the established nesting populations also using the ‘Known’ dataset. For each population, we calculated observed (*Ho*) and expected (*He*) heterozygosities, inbreeding coefficient (F_{IS}) and the allelic richness (Ar) using the ‘*hierfstat*’ package (Goudet 2005) in R software version 4.1.1 (R Core Team 2019). We calculated pairwise differences between baseline populations using Nei's estimator of F_{ST} (Nei 1987) by using the R package ‘*hierfstat*’ (Goudet 2005) and applying 999 permutations to calculate the corresponding *p* values. We plotted the resulting F_{ST} values as a heatmap with the R package ‘*heatmaply*’ (Galili et al. 2018). In parallel, we performed a discriminant analysis of principal components (DAPC) using ‘*adegenet*’ R package (Jombart 2008). We used the *xvalDapc* function, which employs a cross-validation procedure, to objectively optimise the number of PCs retained. Ultimately, we retained five PCs. Additionally, we employed the Bayesian information criterion (BIC) to identify the suitable number of genetic clusters and we produced a density plot with the individual probabilities based on the discriminant functions. Lastly, we calculated the proportion of successful reassignment of each individual to their original population, using the *compoplot* (Jombart and Collins 2015) and the *assignplot* functions of the '*adegenet*' R package (Jombart 2008).

2.5 | Relatedness and Assignment of Individuals With Unknown Origins

To explore kinship relations among the individuals of the established nesting populations and undetermined origin, we quantified shared alleles between sample pairs using the '–relatedness2' function of VCFTOOLS, which follows the approach outlined by Manichaikul et al. (2010). These relatedness metrics were then used to generate a heatmap and a density plot, using the '*ggplot*' package (Wickham 2006) in R software version 4.1.1 (R Core Team 2019).

We used the 'Combined' dataset (Table S5) to perform a Multidimensional Scaling Analysis (MDS) plot based on the 'Identity By State' (IBS) individual pairwise distances obtained with PLINK version 1.07 (Purcell et al. 2007). The first two dimensions resulting from the analysis were plotted using the *ggplot* function from '*ggplot2*' R package. Likewise, we performed the same procedure several times using the datasets 'A', 'B', 'C', 'D', 'E' and 'F' (Table S5), since it has been shown that including highly related individuals in the same MDS plot modifies their position based on the uneven sample sizes and the greater genetic similarities within the nest, as also found in previous studies (Luna-Ortiz et al. 2024). To address this, we retained only one representative from each highly related cluster (i.e., only one randomly selected individual per nest).

We performed Bayesian clustering analyses with STRUCTURE version 2.3.4 software (Pritchard et al. 2000) on the 'Combined' dataset. The program employs a Bayesian clustering technique to assess the probability of association of unique individuals to several potential source populations (Carreras et al. 2011). We ran the program with 1,000,000 Markov chain Monte Carlo (MCMC) iterations and an initial burn-in of 100,000, with 10 independent runs for $K=3$ (the number of the populations in the 'Known' dataset) using an admixture model and assuming independent allele frequencies. CLUMPP version 1.1.2 (Jakobsson and Rosenberg 2007) was used to concatenate the data from the independent runs corresponding to the selected K value.

For a third method of individual assignment, we employed the R package '*assignPOP*' version 1.1.4 (Chen et al. 2018). This program uses a supervised machine-learning approach to assess the discriminatory power of our baseline data to accurately determine the population of origin of the 'Unknown' dataset (Chen et al. 2018). The first step of the '*assignPOP*' approach was the Monte-Carlo cross-validation procedure of the 'Known' dataset. All the reference individuals were randomly split into training and test groups to test the assignment accuracy of the training data. For the F_{ST} loci sample method, we ran 30 iterations of the five available predictive models: support vector machine (SVM), linear discriminant analysis (LDA), Naive Bayes (naiveBayes), Decision Tree (tree) and Random Forest (randomforest). To improve the accuracy values, we ran these models modifying the following parameters: proportion of individuals used as training data (0.5, 0.7 and 0.9) and proportion of training loci used (0.1, 0.25, 0.5 and 1). The best assignation accuracy values were achieved when all loci and 0.9 (90%) of the individuals were used with the SVM predictive model (Figure S1). We included all individuals and polymorphic loci from the 'Known' dataset and the best predictive model (SVM) to determine the likely source population for the individuals included in the 'Unknow' dataset. We ran 10 replicates of this process and calculated the mean of the probability of assignment per individual.

2.6 | Satellite Tracking

We deployed satellite transmitters on GT_1, GT_2, GT_3 and GT_4. For GT_1 and GT_2, we used SPOT-375 A (Wildlife Computers, Redmond, WA, USA). For GT_3, we used a F6G 676 (Lotek, Havelock North, New Zealand) and for GT_4, we used K2G 576E (Lotek, Havelock North, New Zealand). GT_1, GT_2 and GT_3 were spotted in the southwestern Mediterranean (Ceuta, Spain), while individual GT_4 was spotted in the northwestern Mediterranean (La Ràpita, Spain). To deploy these tags, we followed the 'epoxy' method. In brief, the first and second vertebral scutes were cleaned of epibiota, scrubbed to remove excess keratin, and then cleaned with acetone. Finally, the transmitters were attached using a marine epoxy (Araldite). Additionally, we reported the start and end dates of monitoring (Table S3).

All transmitters relayed locational data via the Argos Satellite System (Maryland, USA). To filter out the raw location data and remove spurious locations exhibiting an unrealistic movement speed, we deleted all locations requiring movement speeds exceeding $100\,\text{km}^{-1}/\text{h}$. Next, we used a hierarchical Bayesian state space model (BSSM) to smooth the tracks and provide daily location estimates (see Jonsen et al. 2023). The BSSM was run with two chains for 10,000 MCMC iterations with a 7000 burn-in (thin = 5).

2.7 | Reproductive Status of Satellite Tagged Individuals

All satellite tagged individuals were caught as fisheries bycatch and therefore were examined and treated for injuries resulting from accidental capture before release. The methodology for determining sex and maturity was based on the specific conditions of each individual animal. The sex of adult-sized individuals was first assessed by examination of external characters. This first assessment included biometric measurements (CCL) and the presence of an elongated tail typical of adult males. Additionally, all mature-size individuals received an assessment of their reproductive status through ultrasonography of the caudal coelomic cavity using a General Electric Logiq E Vet ultrasound machine with linear (12LRS) and microconvex (8CRS) probes (GE Medical Systems). Finally, for the female individual (GT_4) with a CCL within reproductive sizes, sex was assessed through hormonal analysis, checking for progesterone, testosterone and 17 β-oestradiol that were analysed in plasma samples from turtle GT_4 using competitive chemiluminescent immunoassay (Snibe Maglumi 600 Analyser).

3 | Results

3.1 | Genomic Analysis

We obtained genomic data from 22 individuals, including 15 'Known' individuals (green turtles from established nesting sites) and 7 'Unknown' individuals (green turtles found outside the typical range for the species in the Mediterranean) (Table S1). However, the individual GT_1 did not yield sufficient DNA to generate a genomic library. From the genomic 2bRAD data, we obtained a total of 117,184,561 raw reads (Table S1) with a mean of 5,326,571 reads per individual (SD ± 1,014,122.3). Filtered reads were mapped against the reference genome for green turtles (GenBank accession GCF_015237465.2), obtaining a high percentage of mapping for all individuals (average value of 92.26% ± 2.14% standard deviation, Table S1). A mean number of 3,937,045.7 mapped reads per individual (SD ± 783,290.4) were used for genotyping, and we identified 60,459 polymorphic variants. After filtering, we retained 11,307 SNPs (Table S1) with a mean (±SD) depth of reads per locus of 22.76 (±8.15). When examining the genomic distribution of SNPs, we observed a strong correlation ($R^2 = 0.993$) between the number of polymorphic loci and chromosome size (Figure S3a). Additionally, the mean depth of SNPs was homogeneous across all chromosomes (Figure S3b).

For the individual GT_1 whose tissue sample presented insufficient DNA for genomic analysis, we were able to obtain its D-loop sequence resulting in haplotype CM-A8.1, prevalent in both the Atlantic (Bjorndal et al. 2006; Encalada et al. 1996; Formia et al. 2006, 2007) and the southwestern Indian (Bourjea et al. 2007), but not in the Mediterranean (Bagda et al. 2012) green turtle rookeries.

3.2 | Genetic Diversity and Population Differentiation of the Baseline

We used the 'Known' dataset to assess the genetic diversity and differentiation among the reference nesting populations constituting the baseline (Akamas, Cayman, Poilão, Table S5) and 11,307 SNPs were retained. The observed (*Ho*) and expected heterozygosities (*He*) and allelic Richness (*Ar*) varied between populations (Table S6), with the highest values found in the Cayman Islands and the lowest values in Poilão. The Cayman population was the only population with an inbreeding coefficient (F_{IS}) significantly different from zero (0.013).

All F_{ST} pairwise distances between the three populations of origin were significantly different from zero (Figure 2a). The lowest value (0.09) was detected between North Atlantic and Mediterranean RMUs and the highest value (0.12) between North Atlantic and South Atlantic RMUs. The DAPC analysis showed three well-defined individual-based clusters associated with the three previously described RMUs (Figure 2b). In addition, Discriminant Functions 1 and 2 had a strong differentiation power of individuals into the three reference groups (Figure 2c). Moreover, the barplot representation of group assignment probability of individuals, extrapolated from DAPC using the BIC approach (Figure 2d), assigned each individual to its population with maximum membership probabilities. We obtained the same result with the graphical representation of the *assignplot* function of the individuals of each baseline population (Figure S4).

3.3 | Origin Assignment of Individuals Sampled

We compiled evidence of four sporadic nesting events of green turtles in western locations out of their nesting range between 2007 and 2021 (Table S2). Mean clutch size per nest was 128 eggs (SD ± 57.08). All of them presented viable hatchlings and the hatching success ranged from 28.3% to 61.3% among the four nests, with a mean of 49.75% (SD ± 0.15%). However, only two provided information on incubation duration (GN2 = 44 days and GN3 = 70 days), as the other nests were identified through emergence of hatchlings (GN1) or were identified weeks after the laying process (GN4). Biological samples were available for all but nest GN4 (Table S2). From one to two individuals per clutch were genotyped with 2bRAD (Table S1). In addition, we collected evidence and tagged four mature-size individuals in the western Mediterranean with satellite telemetry devices (Table S3). However, only three of them (GT_2, GT_3 and GT_4) were included in the genomic analysis due to the poor sample quality of GT_1 resulting in failure to obtain adequate genomic libraries for sequencing.

The relatedness values indicated different levels of relatedness (Figure S5a). The highest relatedness levels correspond to the two samples of the same nest (GN2). The intermediate levels of relatedness correspond to individuals from each baseline population and the 'unknown' individuals assigned to these populations. Finally, the lowest relatedness values were found among individuals of different baseline populations, including those assigned to them. Individuals were considered nonrelated, with relatedness values spanning from −0.2 to −0.1 (beige); individuals from the same population of origin, showed relatedness values generally ranging from −0.08 to 0 (light yellow) and individuals within the same nest (orange) relatedness value was 0.22, consistently exceeding those observed between nests (from 0.04 to 0.1; light orange) (Figure S5b). All individuals were clustered according to their genetic origin based on relatedness values, thus providing additional evidence for assignment. Moreover, we generated a MDS plot based on IBS pairwise distances for all individuals (Figure S6). The two first coordinates separated individuals of the 3 'Known' Atlantic and Mediterranean RMUs and clustered the 'Unknown' individuals within them, although some had more isolated positions (Figure S6). Specifically, the female GT_4 was placed in the same cluster as the Mediterranean RMU individuals, and the female GT_3 and the hatchling from nest GN3 clustered with the individuals from the South Atlantic RMU. All the individuals of the clutches laid in Greece (GN1; GN2) were positioned close but

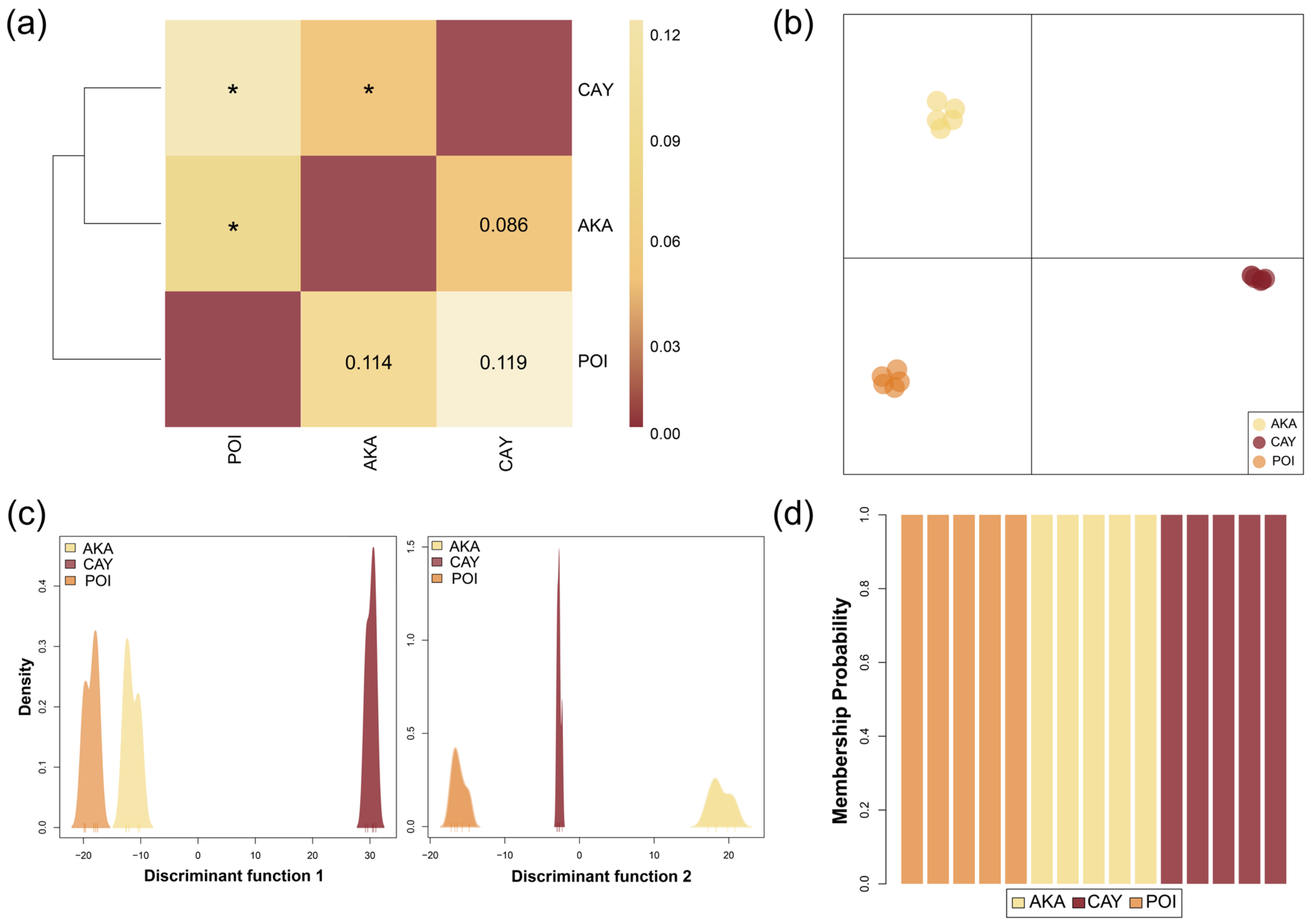

FIGURE 2 | Green turtle population differentiation between Akamas (AKA), Cayman (CAY) and Poilão (POI). (a) Heatmap and dendrogram based on FST pairwise distances. $*p < 0.005$. (b) Discriminant Analysis of Principal Components (DAPC). (c) Density plots of individuals along the first and the second discriminant functions. (d) Assignment population probability of each individual based on DAPC.

not within the Mediterranean RMU group, constituting individualised clusters per nest. Finally, the adult female GT_2 clearly aligned to the South Atlantic RMU, although without overlapping the Poilão samples (Figure S6). No 'Unknown' individual was located in the same cluster or nearby the individuals from the North Atlantic RMU.

Regarding the STRUCTURE approach, we selected $K = 3$ clusters based on the lowest BIC value. Therefore, we selected five principal components and five discriminant functions to represent the DAPC. Regardless of the methodology applied, genomic 2bRAD sequencing assigned all 'Unknown' individuals at RMU level, being the same across methodologies (Figure S7a,b; Table S7). The individuals of nests GN1, GN2 both from Crete and the tagged GT_4 female from La Ràpita (Spain) were assigned to the Mediterranean RMU, the nest GN3 from Tunisia and the mature-size individual GT_2 and GT_3 from Ceuta (Spain) were assigned to the South Atlantic RMU. The probabilities of assignment to the reference populations were high to all individuals (Figure S7). For the MDS with only one 'Unknown' sample at a time, each 'Unknown' sample overlapped completely with the samples of a certain RMU except for GT_2 which did not overlap any RMU cluster although was very close to Poilão (Figure 3). GT_2 was the sample that obtained the lowest probability with '*assignPOP*' (0.72 vs. >0.83 for the other individuals, Table S7).

3.4 | Satellite Tracking

We collected a mean of 391 days (range: 171–626 days) of location data from the four satellite tracked turtles (Figure 4, Tables S3 and S4). Two of the three individuals encountered in the southwestern Mediterranean migrated south along the west coast of Africa before reaching putative foraging areas in Mauritania (GT_1) and Guinea Bissau (GT_3). It is also possible that these mature green turtles were heading to their nesting areas to mate, as Guinea-Bissau is the main nesting site for the species on the Atlantic African coast (Catry et al. 2002). Both were also reported to be of Atlantic origin (South Atlantic RMU) according to genetic assignments. The other turtle (GT_2) encountered in the southwestern Mediterranean migrated east along the northern coastline of Africa until reaching Libya. Genomic analyses also revealed this individual was from the South Atlantic origin. Finally, GT_4 released from the northwestern Mediterranean migrated south to Algeria before heading north

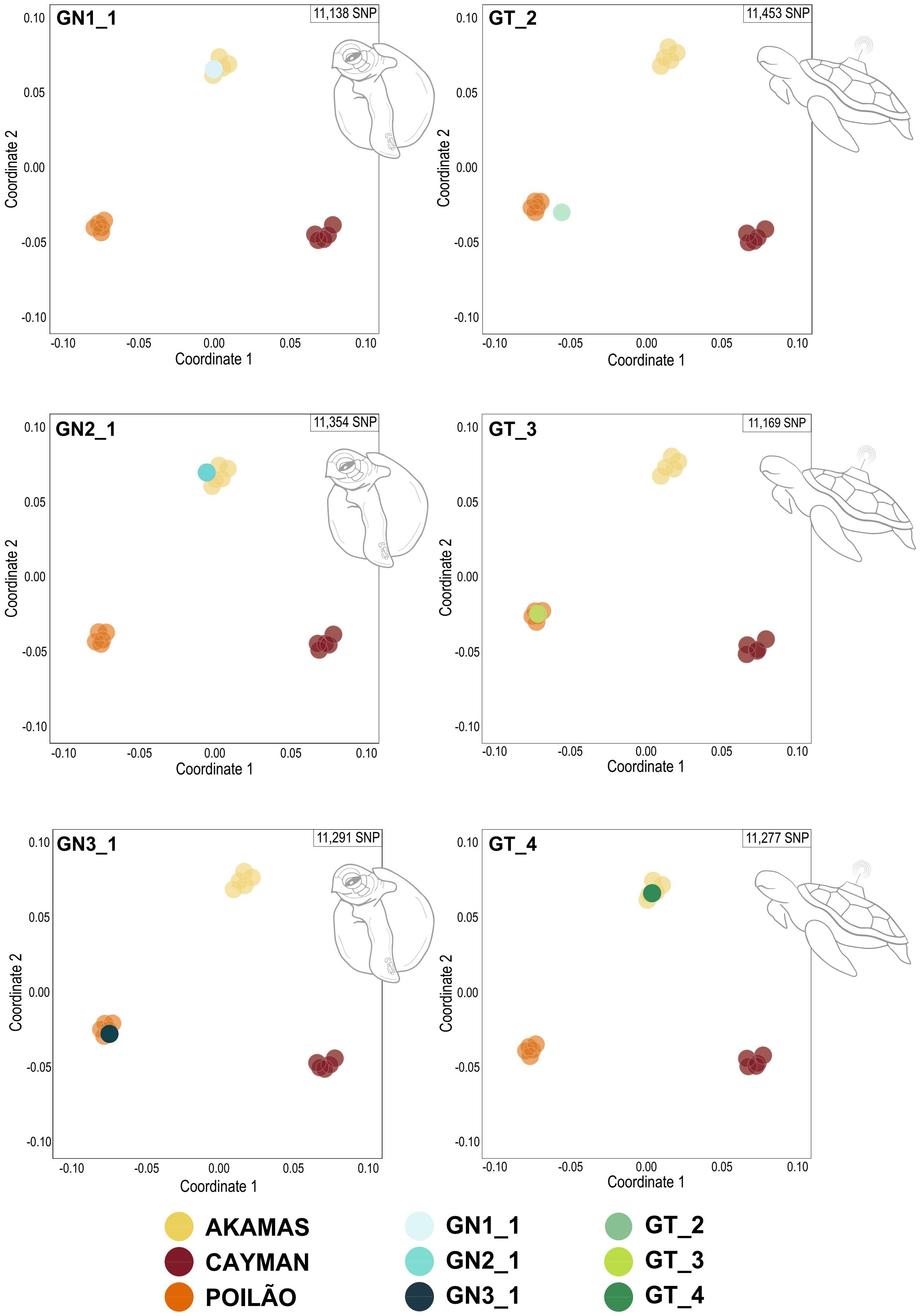

FIGURE 3 | Legend on next page.

FIGURE 3 | Individual plots of multidimensional scaling (MDS) analysis. Each plot represents the datasets 'A', 'B', 'C', 'D', 'E' and 'F' as described in the Table S5. These plots were generated using the green turtle individuals from the established nesting populations (Akamas, Cayman, Poilão), combined with one individual of unknown origin each time. Only one individual per nest was represented, and individuals and populations were coded as in Figure 1.

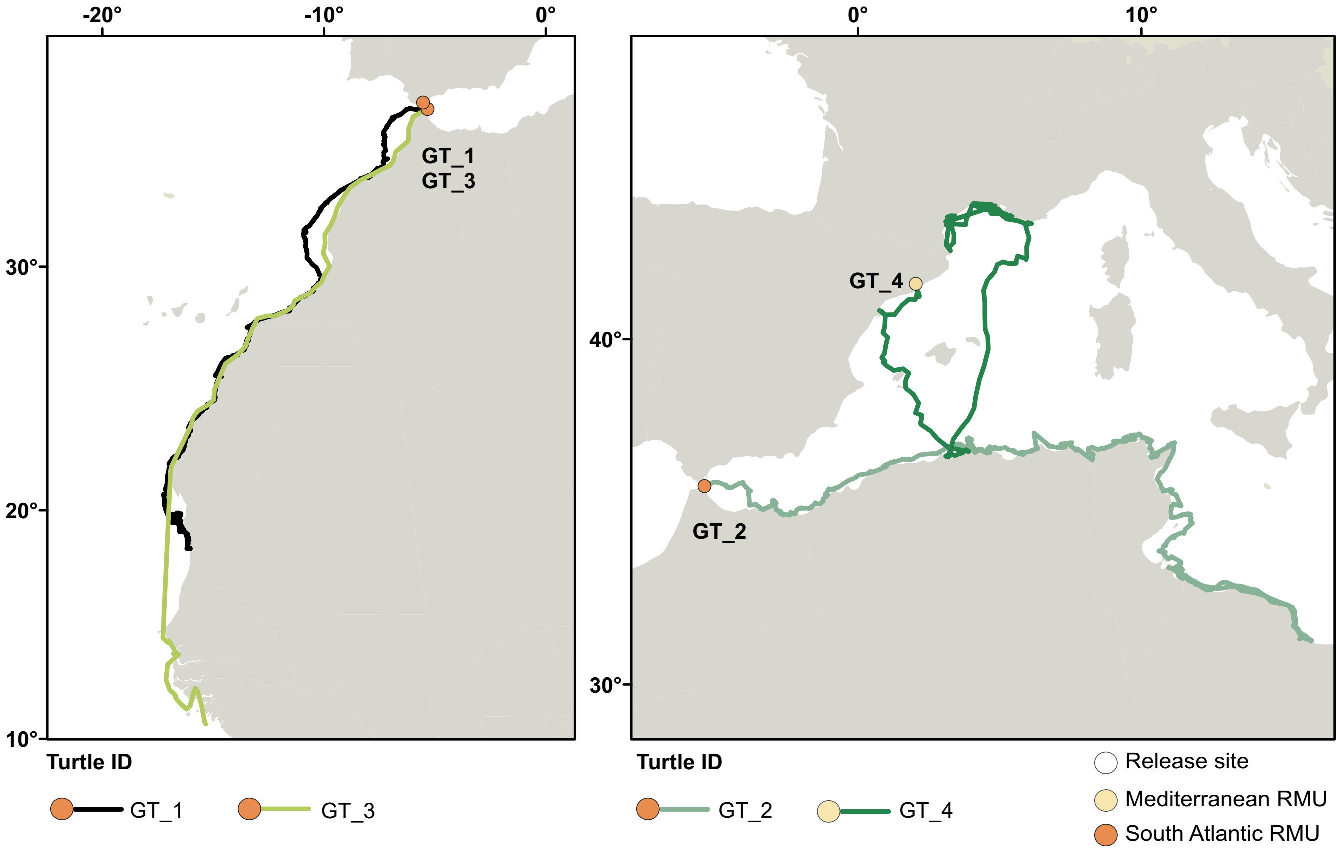

FIGURE 4 | Tracks of four satellite tagged green turtles in the western Mediterranean basin, coloured by turtle ID. The circles represent the release area, and their colour correspond to the RMU (Wallace et al. 2023) of origin of the tagged individuals. Maps were created using ArcGIS version 10.8.2.

to the Gulf of Lion. This individual was assigned genomically to the Mediterranean stock (Figure 4).

3.5 | Reproductive Assessment of Tagged Individuals

GT_1 had an elongated tail, exceeding the size of the posterior fins. Considering the size and tail length, it was considered to be most likely a reproductive male. GT_2 coelomic examination showed signs of an active nesting female. Evaluation through both inguinal fossae showed the abundant presence of follicles of different sizes along the caudal coelomic cavity. Preovulatory follicles were detected (Figure S2a), which could potentially be laid in more clutches during the season, as well as atretic follicles (Figure S2b) (Blanco et al. 2012). Coelomic examination of individuals GT_1, GT_3, and GT_4 did not provide any additional information on their reproductive status.

Biometric data (CCL) was used to determine the mature size of the female GT_3 (112 cm; Table S3), likely being a reproductive female. Finally, hormonal results in turtles GT_4 (Progesterone 1.106 ng/mL, Testosterone 0.057 ng/mL, 17 β-oestradiol 23.54 pg/mL) were indicative of female (Allen et al. 2015; Howell et al. 2022) and within the reproductive size range (73 cm; Table S3) (Sönmez 2019).

4 | Discussion

The integrated results of genomic analysis and satellite tracking shed new light at a fine-scale level on the potential expansion of green turtles in the Mediterranean. Combining both methodologies is crucial for obtaining a comprehensive understanding of their history as genomic techniques reveal the origin of individuals, while telemetry provides information on their onward movement. This allows us to trace both where they come from and where they are headed. Specifically, we revealed that green turtles from both South Atlantic and eastern Mediterranean stocks could play a role in the potential colonisation of the western and central Mediterranean, following a variety of routes once they enter the region. Furthermore, we show for the first time that genomic tools are effective for assigning individual green turtles to specific RMUs.

4.1 | Building a Baseline for Green Turtle Individual Assignments

We developed the first genomic baseline that enabled us to accurately infer the RMU of origin (North Atlantic, South Atlantic and Mediterranean) for unknown green turtles in the Mediterranean. Only with genomic markers, we show the clear differentiation of the three RMUs previously suggested (Wallace et al. 2023). Furthermore, our results suggest that genomics has high potential for individual assignment, even when using a relatively small number of individuals (Bashalkhanov et al. 2009; Nazareno et al. 2017) to establish a baseline for RMU assignment (≥ 5 per RMU).

Interestingly, we detected less genetic differentiation between Mediterranean and North Atlantic RMUs than between either site and the South Atlantic RMU. These results agree with previous global phylogeographic studies of green turtles using mtDNA, where the North Atlantic and the Mediterranean populations were consistently identified as having a shared evolutionary lineage (Encalada et al. 1996; Jensen et al. 2019; Reece et al. 2005). The surface currents and storm events of the North Atlantic Ocean would have favoured this connection (Monzón-Argüello et al. 2012). About 0.7Mya ago, two distinct clades of this species diverged: one encompassing North Atlantic and Mediterranean rookeries, and the other including South Atlantic and West African populations (Encalada et al. 1996; Jensen et al. 2019). Prior to this split, it is likely that the South Atlantic and the Caribbean provided a climatic refugia for Atlantic green turtles (Jensen et al. 2019), similar to the way the Mediterranean served as a refugium for more temperate species such as the loggerhead turtle (Clusa et al. 2013). More recently, the Mediterranean was colonised by green turtles (Encalada et al. 1996) during an interglacial period, where a long-distance dispersal of haplotypes occurred (Encalada et al. 1996; Jensen et al. 2019). The prevalence of widespread haplotypes, as demonstrated by the green turtle in both the Mediterranean Sea (Tikochinski et al. 2012) and the Atlantic Ocean (Carreras et al. 2014), has precluded individual assignment in the species until now. Considering the high potential of genomic data to detect structuring and to discriminate the origin of the individuals, establishing a broader SNP baseline for future studies, including more Mediterranean and Atlantic locations, could improve discrimination within RMUs and resolve individuals with lower assignment probabilities as GT_2.

It is worth mentioning that the observed genetic diversity in the Cayman population is likely the result of its historical origin. After near regional extirpation of the green turtle population in the Cayman Islands, a reintroduction programme was established (Bell et al. 2005). Although the individuals selected for genomic analysis were likely unrelated to the Cayman Turtle Conservation and Education Centre (CTCEC) Ltd. (Barbanti et al. 2019), significant signals of inbreeding were observed. This is likely due to a genetic bottleneck caused by extensive harvesting of wild nesting females (Parsons 1963), with the population nearly becoming extinct by the 1980s (King 1982; Stoddart 1980). The possible genetic admixture with individuals related to the farm could reduce the inbreeding signal of the population in the future.

4.2 | Dispersion From Two Distant Origins

Interestingly, the three different assignment approaches that we applied yielded consistent results, except for the general and individual MDS for the nests GN1 and GN2. For instance, our general MDS (Figure S6) suggest distinct populations of origin for the Crete nests different from the one used to represent the Mediterranean RMU. Consequently, the nests with more than one individual (GN2) generate their own cluster due to the high relatedness. Therefore, we avoid this bias by generating individual MDS. However, including multiple individuals from the same nest is not a problem in the relatedness, structure or '*assignPOP*' analyses. Moreover, the relatedness analysis further supports the assignments, as individuals from different RMUs show low relatedness values, clustering separately. Given the consistency of the multiple approaches and the high probability of assignment of these nests, we can conclude that green turtles from two distant RMUs (Mediterranean and South Atlantic) are starting to nest in new areas within the Mediterranean, with no genetic exchange between them.

The three methodologies supported that the tagged individual GT_4 was from the Mediterranean RMU. This is the first report of an eastern Mediterranean green turtle in the western basin, as prior studies suggested that eastern Mediterranean green turtles migrate as far west as Libya or Tunisia (Godley et al. 2002; Rees et al. 2008). Furthermore, previous reports of juvenile green turtles in the western Mediterranean had an Atlantic origin, implying that individuals from Atlantic and the Mediterranean do not share foraging areas (Carreras et al. 2014). However, this should be continuously monitored in the future, considering the results of the present study. This previous study is consistent with our findings regarding tagged adult-sized individuals found in the southwestern Mediterranean, as they were all assigned to the South Atlantic RMU. While GT_3 was attributed to the South Atlantic RMU with high accuracy in all analyses, GT_2 had lower assignation probabilities and was plotted nearby but not within the South Atlantic RMU in the individual MDS analysis. This result suggests that the 'Known' dataset, which included individuals from a single nesting location per RMU, may not fully capture the genetic diversity within RMUs. Consequently, it is possible that GT_2 belonged to a different Atlantic subpopulation such as the one at Ascension Island, which was estimated to be genetically similar to Poilão based on the D-loop haplotype (Patrício et al. 2017) but may have some genomic differences in terms of nuclear DNA. Although the main objective of this study is to achieve assignments at the RMU level, incorporating individuals from additional populations in future research could provide valuable insights into intra-RMU structuring. Finally, although genomic analysis was not possible, the individual GT_1 could also be assigned to Atlantic origin based on mtDNA. This individual exhibited the haplotype CM-A8.1, identified in the Atlantic (Bjorndal et al. 2006; Encalada et al. 1996; Formia et al. 2006, 2007) and southwestern Indian ocean (Bourjea et al. 2007). This haplotype is particularly prevalent in west African nesting rookeries, such as the Poilão and the Ascension Island populations (Formia et al. 2006; Patrício et al. 2017). Consequently, using the mtDNA haplotype approach, this individual could potentially belong to the

South Atlantic, South Central Atlantic, Southwest Atlantic or Southwest Indian RMUs (Wallace et al. 2010). Thus, the precision achieved with our genome-wide SNP baseline significantly surpassed the accuracy of previous studies, as the origin RMU was in all samples assigned with high probability, indicating the great potential of this approach for future studies relying in individual assignments. The case of GT_1 exemplifies the limitations of broad mtDNA-based assignment (encompassing both the Atlantic and Indian Oceans) and highlights the value of using 2bRAD to achieve more accurate and reliable population assignments.

Telemetry data complements our genetic analyses by showing the migratory routes followed by the turtles after sampling. The route of the female GT_4 in the western Mediterranean towards the Algerian coast and backwards to the Gulf of Lion is a new, previously undescribed movement for green turtle, as previous studies reported movements in the Eastern Mediterranean (Bradshaw et al. 2017; Godley et al. 2002; Rees 2013; Stokes et al. 2015). Continued monitoring of adult individuals will determine whether this is an isolated event due to the disorientation of this specific individual or a new route described for green turtles in the Mediterranean, as extensive previous studies have never documented similar routes for Mediterranean individuals (Casale et al. 2018 and references therein). Two of the turtles belonging to the South Atlantic RMU released in the southwestern Mediterranean, GT_1 (male) and GT_3 (female), moved towards the Atlantic along the west coast of Africa, consistent with green turtles' highly philopatric behaviour (Catry et al. 2002). The tagged turtles were mature-sized, suggesting a minimum size may be required for their return to Atlantic waters. A study combining genetics and experimentation indicated that loggerhead turtles need to reach a certain size to swim against the prevailing currents and exit the Mediterranean into Atlantic waters (Revelles et al. 2007), which is probably also the case for green turtles. Nevertheless, heterogeneity in the size of the tracked individuals would be required to study if their distribution and movements are size-dependent as occurs with the loggerhead turtle (Eckert et al. 2008). Additionally, despite its Atlantic origin and size, the female GT_2 also released in the southwestern Mediterranean remained within the Mediterranean Sea, navigating along the northern coast of Africa and reaching the coast of Tunisia—where nest GN3, of Atlantic origin, was found. Interestingly, based on ultrasound examination of subject GT_2, it was probably bycaught during the internesting period, as she was ready to lay eggs. Although this female did not lay nest GN_3, its movement revealed the routes followed by some Atlantic adult individuals probably during their feeding period. Consequently, adults remaining in these warmer foraging grounds might suffer accelerated gonad development due to increased temperature. This fact could lead them to experience the urge to mate and consequently lay their nests before returning to their natal populations. This hypothesis could explain the presence of nests outside their typical range and near foraging grounds.

The relatedness analysis revealed that none of the nests were laid by the mature-size individuals sampled in this study, suggesting that there are other reproductively active green turtles in the western and central Mediterranean. Additionally, the analysis confirmed that the two nests laid in Crete were produced by different progenitors. The newfound insights into the origin and routes of these green turtles are highly pertinent for consideration of the implementation of conservation actions. These measures should focus on protecting nesting beaches that will be suitable in the future (Patrício et al. 2021), which may not be considered important nowadays due to the nesting being scarce and sporadic. For instance, the recent colonisation of the loggerhead turtle in the western Mediterranean has directly influenced species management plans in Italy, France and Spain, demonstrating the practical applications of such research in conservation efforts (Hochscheid et al. 2022).

4.3 | Future of the Green Turtle Colonisation in the Mediterranean

Our results suggest that we may be witnessing early signs of a shift in the foraging and breeding distribution of the green turtle in the Mediterranean. Modelling under various climate change scenarios predicts an increase in nesting, with higher probabilities in the western Mediterranean (Arslan et al. 2023; Mancino et al. 2023). Our results suggest an incipient colonisation by this species in new areas of the Mediterranean Sea, originating from distant regular nesting areas from the South Atlantic and Eastern Mediterranean RMUs (Wallace et al. 2023). Future colonisation progress can only be speculated, based on reproductive parameters from reported nests, predicted warming and the colonisation of other sea turtle species in the region (Carreras et al. 2018; Luna-Ortiz et al. 2024; Maffucci et al. 2016; Mancino et al. 2022; Tomás et al. 2008).

All reported nests produced viable hatchlings indicating some potential for the establishment of new populations. The clutch size variation from 62 to 198 eggs is consistent with observations in this species (Hirth 1997). Furthermore, viable hatchlings were produced in all nesting events, with hatching success rates ranging from 28.3% to 61.3%.

It has been hypothesised that the colonisation process of the loggerhead turtle in the western Mediterranean is related to the early sexual maturation of individuals from nearby foraging areas induced by high temperatures (Cardona et al. 2023; Carreras et al. 2018; Luna-Ortiz et al. 2024; Santidrián Tomillo et al. 2024). Considering that the green turtle has more tropical preferences than the loggerhead turtle (Seminoff et al. 2015), they may follow a similar colonisation pattern, but with some time delay owing to the different temperature preferences. Recent modelling studies of nesting suitability for green turtles suggest that future optimal conditions will be moving to the central and western Mediterranean (Arslan et al. 2023; Mancino et al. 2023). Linked to the newly found nesting events, the recent detection of mature-size green turtles in the western basin, as reported in the present study, is a novel and significant finding. This implies the presence of reproductively mature individuals in this region where only juvenile green turtles were previously reported (Bentivegna et al. 2011; Carreras et al. 2014). Future monitoring is essential to assess the progression of this potential colonisation, and the effects of global warming on species viability in these new areas. Incorporating advanced next-generation sequencing techniques will provide a clearer insight on both nests and mature-size individuals, offering a better understanding of the current and future status of individuals from different RMUs coexisting in the Mediterranean waters. Our results also have to be taken into consideration for the management and conservation of this endangered species in the central and western Mediterranean.

Author Contributions

C.C. conceptualised and designed the study. J.L.C.-P., L.G., A.P., R.P., S.D., D.M., J.H. and I.J. obtained the samples. G.M.C. did the laboratory procedures. G.M.C. and C.P. did the genotyping and genomic data analyses with inputs from M.P. and C.C., J.L.C.-P. placed the satellite devices on the tagged individuals and conducted the reproductive assessment status of the adult-sized individuals. N.J.R. analysed the satellite telemetry data. G.M.C. did the illustrations of the manuscript. G.M.C., M.P., C.P. and C.C. wrote the manuscript with input from all authors.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

2bRAD raw data are available at the European Nucleotide Archive (ENA) under the project PRJEB70796.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.