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Mitochondrial DNA short tandem repeats unveil hidden population structuring and migration routes of an endangered marine turtle

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Abstract

1. The assessment of the composition and dynamics of endangered populations is crucial for management and conservation, and appropriate genetic markers are critical.
2. The genetic structuring of the Mediterranean green turtle (*Chelonia mydas*) populations and the origin of the stranded animals found along the Israeli coast was investigated using new highly polymorphic short tandem repeat (STR) markers.
3. The structuring of nesting populations was studied using pairwise genetic distances and a principal coordinates analysis (PCoA).
4. The contribution of the different nesting populations to the stranded sample was assessed by using a mixed-stock analysis.
5. A clear population genetic structure, not detected before, has been revealed. The four nesting populations are genetically well differentiated, and thus should be considered as different management units. The populations from Turkey and Israel showed higher resemblance, despite residing at opposite ends of the Mediterranean distribution. The Turkish nesting population is the main source of the stranded turtles sampled along the Israeli shore, confirming that individuals from this population migrate from north to south along the eastern shore of the Mediterranean, as previously shown by telemetry studies.
6. The use of a highly polymorphic haplotyping method enabled the detection of a clear genetic structuring of the green turtle populations in the eastern Mediterranean Sea that was not revealed in previous studies, demonstrating the importance of marker selection in population genetics.
7. The analysis of the genetic composition of the stranded turtles allowed us to investigate the migration patterns from nesting to foraging areas, supporting previous satellite-tracking and stable-isotope results.
8. These results will help to delineate conservation management units for the species in the Mediterranean, and reveal connectivity among beaches and mixed aggregations.

KEYWORDS

Chelonia mydas, genetic diversity, Mediterranean Sea, mitochondrial DNA, mixed-stock analysis, short tandem repeats

1 | INTRODUCTION

The assessment of the structuring of populations and their connectivity with the relevant foraging areas are key elements for the management and conservation of marine organisms such as marine turtles (Rees et al., 2016). A homogeneous and well-connected network of populations would interchange individuals, and therefore any local threat, management, or conservation action would affect the whole network. On the other hand, restricted connectivity among populations demands localized efforts, as each independent management unit would not benefit from actions undertaken in other locations (Moritz, 1994). The assessment of structuring and connectivity is especially challenging in highly migratory species, as they move across vast distances using different areas to develop, reproduce, or forage. For instance, a specific threat could be highly localized within a foraging area, yet could potentially impact a number of different distant reproductive areas (e.g. Clusa et al., 2016). A wide variety of methodologies has been used to assess both population structuring and connectivity among different habitats used by individuals, dependent upon the resources available, the specific research questions, and the biology of the studied species. Among those, genetics has been the preferred method to evaluate population structure. The levels of connectivity among populations can be inferred through the degree of genetic differentiation retained as a result of isolation (Matsuzawa et al., 2016; Roden et al., 2013), and can also reflect the historical processes that shaped the actual distributions (Clusa et al., 2013). Contemporary movements of individuals can also be tracked using tag–recapture techniques (Rees et al., 2013), satellite telemetry (Stokes et al., 2015), or stable isotopes (Hobson, 1999), thus linking reproductive areas with developmental and foraging areas. Nevertheless, these techniques are not exempt from some limitations. Satellite tracking is the most direct way to follow individual turtles; however, sample sizes are necessarily limited because of the high implementation costs, and studies are often undertaken over short time periods owing to technological restrictions (Hebblewhite & Haydon, 2010). Tag–recapture techniques depend on obtaining a significant number of recaptures, which is a challenging task in populations of highly dispersed animals (Revelles et al., 2008). Stable isotopes rely on the discriminatory power of the markers used in the different foraging areas and robust baselines (Hobson, 2008). Finally, the selection of the appropriate genetic marker is crucial when planning a scientific project (Karl, Toonen, Grant, & Bowen, 2012). For these reasons there are also several examples of unresolved case studies arising from methodological limitations, despite the research efforts.

One of these unresolved case studies is the green turtle in the Mediterranean Sea. This area is the nesting habitat of two of the seven sea turtle species, the loggerhead turtle (*Caretta caretta*) and the green turtle (*Chelonia mydas*). Whereas the loggerhead population has increased in the past decade (Casale & Margaritoulis, 2010), the Mediterranean green turtle is still declared endangered by the International Union for Conservation of Nature (IUCN), 2016), although recent studies suggest that populations are now increasing (Stokes et al., 2014). Mediterranean green turtles nest only in the eastern basin, from east Turkey through Cyprus and the Levant shoreline to eastern North Africa (Egypt) (Casale & Margaritoulis, 2010), and it is estimated that there are about 1350 nesting females in the whole area (reviewed in Stokes et al., 2015). Despite these numbers, they are remnants of much bigger populations that existed until the beginning of the 20th century (Bjorndal, 1995; Kuller, 1999). As this species is known to have very high levels of female philopatry (Miller, 1997), high levels of population structuring are expected when maternally inherited genetic markers are used for analysis (Meylan, Bowen, & Avise, 1990). This has been confirmed by different studies using the mitochondrial control region (D loop) as a marker, showing well-structured populations worldwide (Encalada et al., 1996; Naro-Maciel et al., 2014); however, several studies have highlighted the lack of resolution of the mitochondrial DNA control region sequence used for the species when applied to the Mediterranean green turtles, as a result of the over dominant presence of a single haplotype, CM-A13 (Bagda, Bardakci, & Turkozan, 2012; Kaska, 2000; Naro-Maciel et al., 2014). This lack of resolution has undermined the definition of the management units present in the area, and has thus impacted on the management and conservation efforts of the species.

The lack of resolution of the genetic markers previously used in this species has also prevented the accurate determination of the origin of the turtles present in the Mediterranean foraging grounds. Marine turtles are highly migratory animals and foraging grounds usually host mixed aggregations of individuals from different nesting populations (Bowen & Karl, 2007). Mixed-stock analysis (MSA) was first developed to assess the stock origin of mixed aggregations of fishes (Grant, Milner, Krasnowski, & Utter, 1980; Pella & Masuda, 2001; Pella & Milner, 1987), before being applied to other highly migratory marine organisms like marine turtles (Bass, Epperly, & Braun-McNeill, 2004). It has been used to assess migratory routes (Carreras et al., 2006), foraging grounds (Jensen, Pilcher, & FitzSimmons, 2016; Rees et al., 2017), and the impact of threats in common foraging grounds on source populations (Clusa et al., 2016). Accurate MSA relies on the power of the genetic markers used to detect structuring among the nesting populations, however, and for this reason no attempt has been made, to date, to evaluate the composition of the known foraging aggregations of Mediterranean green turtles. Several episodes of green turtle local mortality have been reported in the Mediterranean, for which the origin of the affected individuals has not yet been resolved. For instance, thousands of green and loggerhead turtles were harvested during the Second World War along the eastern Mediterranean coasts

(Levy, 2010; Levy et al., 2015; Sella, 1982). This episode contributed to the general decline of the Mediterranean populations, although the precise distribution of this impact on source populations is yet unknown. More recently, Israeli shores have been subject to a large number of stranded turtles, live individuals of which were brought to the rescue centre for recovery (Levy, 2010). The Israeli green turtle nesting population is estimated at fewer than 20 nesting females, according to the Israeli Nature Parks Authority, with a current average of eight green turtle nests per year: a nesting abundance that is much lower than in the recent past (Levy, 2010). Thus, this tiny nesting population is unlikely to explain the large numbers of stranded individuals, implying that some of these stranded turtles originate from other populations. Therefore, genetic markers with greater resolution are necessary to assess the geographical origin of the individuals visiting this foraging area.

New promising mitochondrial markers have been tested in Mediterranean green turtles (Tikochinski et al., 2012). As repeat regions undergo mutational changes much faster than unique sequences through slipped-strand mispairing or unequal recombination (Tautz & Schloetterer, 1994), greater polymorphism can be found in these regions, thereby establishing the basis for short tandem repeat (STR) analysis in genomic DNA. This haplotyping method looks at the 'AT' repeat region in the 3′ end of the control region of the mitochondrial DNA, as it contains four different STRs separated by short spacers. This new haplotyping method revealed 33 Mediterranean haplotypes, enabling detailed genetic analysis of the Israeli green turtle nesting population and stranded individuals, and has already assisted in discovering 'cryptic diversity' in several studies in other geographical regions (Shamblin et al., 2015). These STR markers have not yet been applied to decipher the population structuring among the Mediterranean green turtle populations, however, nor have they been used to undertake an MSA in any marine turtle study.

In order to look at the dynamics of the whole Mediterranean green turtle population, a mitochondrial DNA (mtDNA) STR haplotyping method was used to analyse the four major nesting areas located in Turkey, Akamas, Alagadi, and Israel. This work also aimed to decipher the origin of the stranded individuals along the Israeli coast, to assess the populations that are using this foraging area. The data presented here provide the first regional fine-scale assessment of Mediterranean green turtle population structuring, as well as the principal migratory paths unveiled by this and previous studies.

2 | METHODS

2.1 | Data collection

2.1.1 | Sampling

Green turtle nesting females or dead hatchlings were sampled between 2001 and 2015 at nine sites along the Mediterranean coastline of Cyprus (Akamas and Alagadi), eastern Turkey (Anamur, Göksu Deltasi, Alata, Kazanlı, Akyatan, and Samandağ), and Israel, along the coastline between Betzet and Ziquim (Figure 1; Table S1). Additionally, samples were collected from dead and live stranded turtles in the same section of the Israeli coast (Figure 1; Table 1). Stranded turtles were measured in order to infer life stage, when possible (curved carapace length; CCL in cm). Tissue samples, comprising a small skin biopsy (<0.5 cm^2), were taken from the membrane on the trailing edge of the forefront flipper. When no tissue was available from a nesting female, two or three dead hatchlings were collected from each nest to guarantee a successful DNA extraction, although only one hatchling per nest was used for further analyses using the common maternal haplotype. All nesting females were tagged shortly after laying to avoid pseudoreplication (Broderick, Glen, Godley, & Hays, 2002; Stokes et al., 2014), with the exception of Akamas samples, where only 23 nest samples could be unambiguously considered independent according to the nesting date and the remigration interval of the species (Table 1). Storage conditions and DNA extraction procedures varied among sampling sets, as they were performed by different research teams (Table S2).

2.1.2 | Haplotyping

A 200-bp fragment of the 3′ end of the mtDNA control region was amplified using the primer pair CM-D-1 F (5′-AGCCCATTT ACTTCT CGCCAAACCCC-3′) and CM-D-5 R (5′-GCTCCTTTTATCTGATGGG ACTGTT-3′) (Tikochinski et al., 2012). Polymerase chain reactions (PCRs) and PCR conditions varied among sampling sets, as they were performed in different laboratories (Table S2). PCR products were visualized by electrophoresis to ensure successful amplification, and a total of 6 µl of the PCR product was purified using 2 µl of ExoSAP-IT® (Affymetrix Inc.), following the manufacturer's instructions. The purified mtDNA amplicon was sequenced in forward and reverse directions in an ABI 3730 DNA Analyser (Applied Biosystems™), or was sent to the sequencing laboratories of Macrogen®. All PCR reactions were run with positive and negative controls. Sequences were edited and aligned using GENEIOUS 6.17 (Biomatters Ltd) or BIOEDIT 7.2.5 (Hall, 1999). STRs were scored by counting the number of 'AT' repeats in each of the four loci of the sequence, and haplotypes were defined by combining the four STRs and named using the four-number barcoding system described in the literature (Tikochinski et al., 2012). In cases of heteroplasmy of the mtSTRs, the major haplotype was taken, based on the relative peak heights, as previously described (Tikochinski et al., 2012). For all of the analyses, each 'AT' repeat was treated as a single mutation event and indels were added when necessary in order to obtain sequences of equal length. Furthermore, because of the nature of the polymorphism, only frequency-based statistics were used.

2.2 | Data analysis

2.2.1 | Population structure

The haplotype diversity (*H*) for each population was calculated and tested for population structuring by calculating pairwise genetic distances (F_{ST}) and performing exact tests of population differentiation, as implemented in ARLEQUIN 3.5 (Excoffier & Lischer, 2010). All multiple comparisons were corrected using a false discovery rate (FDR) approach (Narum, 2006). Pairwise F_{ST} values obtained from ARLEQUIN were then used to perform a principal coordinate analysis

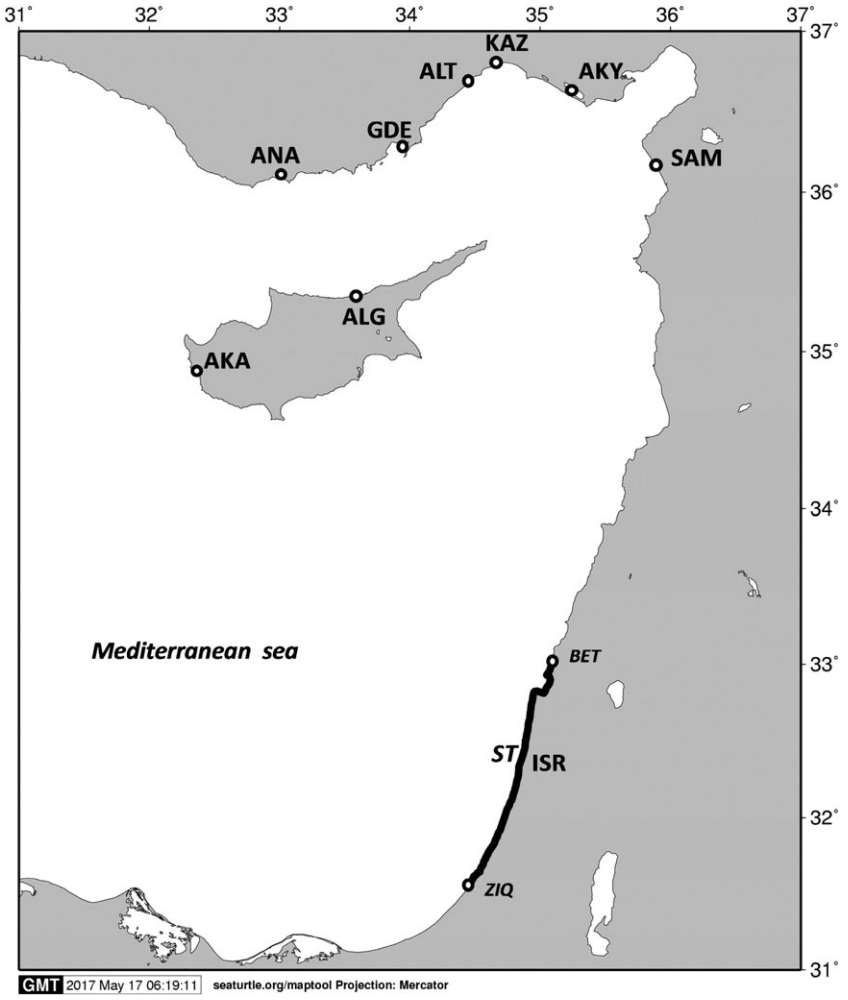

FIGURE 1 Sampling locations. Circles show the nesting sites that were sampled in our study. The black thick line represents the geographical range of the coastline were the stranded animals (*ST*) were obtained. Nesting locations: AKA (Akamas), ALG (Alagadi), ANA (Anamur), GDE (Göksu Deltasi), ALT (Alata), KAZ (Kazanli), AKY (Akyatan), SAM (Samandag), and ISR (Israel). All Israeli samples were collected between the locations of BET (Betzet) and ZIQ (Ziquim). For statistical analyses, the nesting sites from Turkey were grouped as TUR (Turkey, composed of ANA, GDE, ALT, KAZ, AKY, and SAM). See main text and Table S1 for details. Map created using the free software MAPTOOL (SEATURTLE.ORG, Inc. http://www.seaturtle.org/maptool/)

(PCoA) in GENEALEX 6.5 (Peakall & Smouse, 2012) in order to distribute the variability found across two-dimensional space.

2.2.2 | Mixed-stock analysis

A Bayesian MSA was used to assess the composition of the Israeli stranding stock through the use of Bayes (Pella & Masuda, 2001). This analysis estimates the proportion of individuals of the mixed stock coming from the different nesting populations; the Mediterranean nesting populations sampled in the present study were used as the baseline. Three different simulations were performed: (i) no weighting factor; (ii) using an estimate on the size of each rookery (expressed as the mean number of nests per year; Table 1) as a weighting factor, as suggested by previous studies (Bass et al., 2004); and (iii) using the minimum dis tance across sea (expressed in km) as a weighting factor. Population sizes were taken from the literature (Stokes et al., 2015), and the minimum distance across the sea was measured using GoogleEarth®. Iterated chains were considered reliable when the Gelman–Rubin criterion was fulfilled (i.e. with a GR shrink factor of <1.2 for all parameters), as described in the software manual.

3 | RESULTS

Thirty-seven different haplotypes were found among all samples, nine of which were exclusive to the stranded individuals (Tables 1 and S3). The small sample size from Israel is the result of the low number of nesting females scattered across the region (Table 1), meaning that potentially almost all of the individuals in this population according to the last census size were sampled (Table S1). All samples from Turkey were pooled and coded as TUR for the remaining analysis because of the low sample sizes obtained from some of the sites, and as no significant pairwise comparison was found among all locations (global F_{ST} = −0.012, p = 0.639). Finally, not all samples of Akamas could be unambiguously considered to belong to independent females (Table 1), as only 23 of the 69 sampled nests were unambiguously independent, taking into account the nesting date and the remigration interval of the species. Thus, the population structure analyses were performed using both the filtered (including the Akamas samples without risk of pseudoreplication, n = 23) and unfiltered (including all Akama samples, n = 69) data sets. The analyses including the independent and the whole data sets yielded similar results (Table 2), with the distances among all populations obtained from both analyses being strongly correlated (R^2 = 0.99, P < 0.05; Figure S1). Furthermore, the genetic distance between the two data sets from Akamas did not significantly differ from zero (F_{ST} = −0.023, P = 0.97). Therefore, we assumed that the complete data set was free from any pseudoreplication bias, and as a consequence it was used for the following analyses in order to maintain the haplotypes found at low frequencies. The data set used for the following analyses is detailed in Table 1.

TABLE 1 Absolute mtDNA haplotype frequencies found in the four nesting populations and the Israeli stranded *Chelonia mydas* turtles

	AKA	ALG	TUR	ISR	ST	Total
Population size	48	66	572	18*	–	
Haplotype diversity (*H*)	0.406 (0.3834)	0.824	0.841	0.993	–	
Haplotype						
6–8–8-4	53 (18)	23	17	2	49	144
6–8–5-4	3 (1)	6	27	1	62	99
6–8–6-4	5 (3)	32	11	1	17	66
6–9–6-4	0	28	1	1	5	35
8–7–7-4	0	0	6	1	19	26
6–8–7-4	1	1	8	1	10	21
7–8–7-4	1	7	0	0	2	10
7–8–8-4	1	2	1	0	5	9
7–8–5-4	0	1	3	1	4	9
6–8–9-4	0	1	0	1	7	9
7–8–6-4	0	2	1	0	4	7
7–7–7-4	0	0	3	1	2	6
7–7–6-4	0	0	2	0	4	6
5–8–5-4	0	0	0	1	4	5
7–10–6-4	0	5	0	0	0	5
6–7–5-4	0	1	2	0	2	5
6–9–8-4	2(1)	1	2	0	1	6
6–7–6-4	0	1	1	0	3	5
5–8–6-4	0	1	0	0	4	5
5–8–8-4	0	0	0	1	3	4
8–7–6-4	1	0	2	1	2	6
7–7–8-4	0	0	0	0	5	5
7–9–6-4	0	2	0	0	1	3
8–8–7-4	0	0	0	0	2	2
7–10–5-4	0	0	0	0	3	3
6–8–10-4	0	0	0	0	2	2
6–8–5-5	0	0	0	0	2	2
8–8–8-4	0	0	0	1	0	1
7–9–7-4	0	0	0	1	0	1
6–7–7-4	0	0	0	1	0	1
5–9–6-4	2	0	0	1	0	3
6–11–5-4	0	1	0	0	0	1
6–9–7-4	0	1	0	0	0	1
8–8–5-4	0	0	0	0	1	1
6–10–5-5	0	0	0	0	1	1
5–8–7-4	0	0	0	0	1	1
5–7–6-4	0	0	0	0	1	1
TOTAL	69 (23)	116	87	17	228	517

Nesting areas are defined after grouping, as explained in the text: AKA (Akamas), ALG (Alagadi), TUR (Turkey), ISR (Israel), and ST (Israeli stranded turtles). Numbers in parenthesis indicate the results obtained with the subset of samples that were unambiguously independent from the Akamas population. Population sizes of the nesting areas are expressed as mean nests per year, as found in the literature (Stokes et al., 2015). *Population sizes from Israel were provided by YL as estimated by the Israeli National Sea Turtle Rescue Centre, the Nature and Parks Authority of Israel.

The haplotype diversity was very variable among populations, ranging from 0.994 from Israel to 0.406 from Akamas (Table 1). All four nesting areas were highly differentiated (mean F_{ST} = 0.2024, range 0.0228–0.3153; Table 2), although the degree of differentiation was not the same among all pairwise comparisons. This differentiation was corroborated by the PCoA (Figure 2), which explained an accumulated 96.03% of the variability found along its two axes. Thus, Akamas was the most differentiated population, probably because of the predominance of the 6–8–8-4 haplotype, followed by Alagadi that also presented a high frequency of 6–8–6-4 and 6–9–6-4 (Table 1). Turkey and Israel were the two closest populations in terms of genetic distance, although still significantly differing from each other.

TABLE 2 Pairwise comparisons among eastern Mediterraean nesting populations of *Chelonia mydas*

	AKA	ALG	TUR	ISR
AKA	0	***0.2912***	***0.2555***	***0.2382***
ALG	***0.3153***	0	***0.0930***	***0.0758***
TUR	***0.2870***	***0.0930***	0	***0.0228***
ISR	***0.2900***	***0.0758***	***0.0228***	0

Above the diagonal, genetic distances (F_{ST}) obtained using only the unambiguously independent sample set from Akamas; below the diagonal, genetic distances using all samples. Values in bold indicate significantly different population pairs according to the exact test and after the FDR correction (for a threshold of $P < 0.05$, FDR = 0.0205). Nesting areas: AKA (Akamas), ALG (Alagadi), TUR (Turkey), and ISR (Israel).

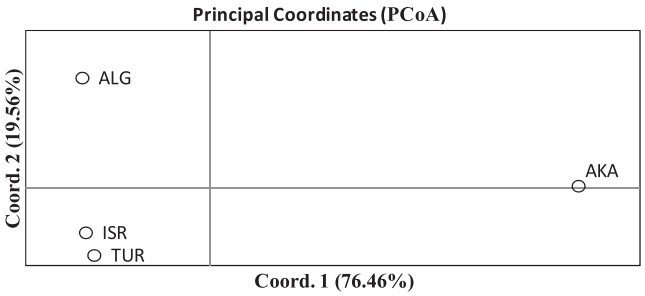

FIGURE 2 Principal coordinate analysis (PcoA) based on the genetic distances (F_{ST}) among the four nesting populations of *Chelonia mydas*. The percentage of variation explained by each axis is shown in brackets. Nesting areas: AKA (Akamas), ALG (Alagadi), TUR (Turkey), and ISR (Israel)

The samples from the stranded individuals included 30 of the 37 haplotypes, nine of which were exclusive and hence could be considered as 'orphan haplotypes' (i.e. haplotypes not found in any of the possible source populations); however, these 'orphan haplotypes' only represented 7.89% of the stranded individuals. The size of the individuals was obtained from 154 of the 228 turtles sampled (Table S4). Haplotype frequencies were compared among three size classes: juveniles (CCL < 30 cm), sub-adults (30 < CCL < 70 cm), and adults (CCL > 70 cm). No significant differences were found among any of the life stages, and for this reason the whole set of 228 samples was used for further analyses (Table S5). The MSA results were very similar regardless of the weighting factor applied (Figure 3). Most of the stranded individuals originated in Turkey, despite being the farthest nesting population, with a smaller contribution from Israel and negligible contributions from the Alagadi and Akamas populations.

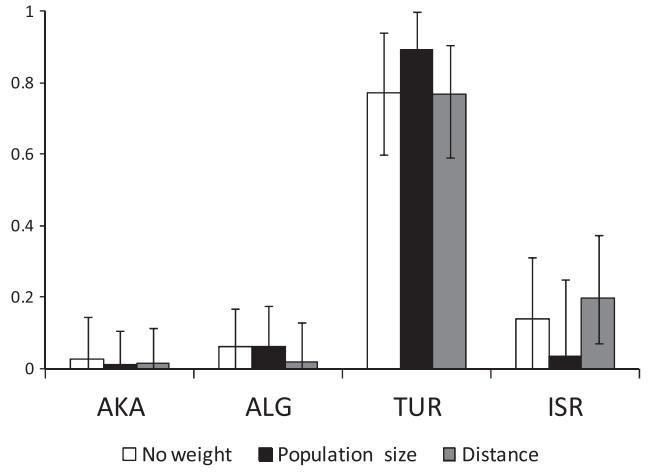

FIGURE 3 Mixed-stock analyses (MSA) of the stranded turtles found along the Israeli coast. Each bar set represents the percentage of turtles that originate from each one of the four groups of nesting populations. Three different analyses were run, including: (i) no weighting factor; (ii) population size as a weighting factor; and (iii) distance to the nesting area as a weighting factor. Error bars show the 95% confidence intervals. Nesting areas: AKA (Akamas), ALG (Alagadi), TUR (Turkey), and ISR (Israel)

4 | DISCUSSION

An understanding of the dynamics of the endangered green turtle population in the Mediterranean was achieved by the use of a highly polymorphic set of markers. The philopatric behaviour of the nesting females makes the maternally inherited mtDNA the appropriate molecule for this analysis (Meylan et al., 1990); however, the resolution of the specific mtDNA marker used, and thus its potential to detect genetic structuring, is highly dependent on its polymorphism. The Mediterranean green turtle was previously considered to host low levels of polymorphism using either a ~380-bp or a ~800-bp mtDNA sequence of the control region, and all of the populations were defined as being genetically homogeneous (Bagda et al., 2012; Encalada et al., 1996). Our novel mitochondrial DNA control region STR analysis revealed much higher levels of polymorphism, with up to 37 different haplotypes, and consequently allowed the detection of unprecedented levels of population genetic structuring within the region. This deep genetic structuring made possible the implementation of robust MSA based on mtDNA STRs, pioneering a new method in marine turtle research.

The lack of genetic structuring found in previous studies in the Mediterranean region using mtDNA was attributed to the recent colonization of the region, coupled with the fact that this molecule evolves far more slowly in marine turtles than in other vertebrates (Avise, Bowen, Lamb, Meylan, & Bermingham, 1992). The green turtle colonization of the Mediterranean probably occurred within the last 10 000 years (Bowen et al., 1992), and the region was likely to have first been colonized by the more temperate loggerhead turtle (*C. caretta*) (Clusa et al., 2013). Thus, it was suggested that mtDNA markers were potentially less accurate for revealing differentiation at finer geographical scales or among recently diverged populations (Formia, Godley, Dontaine, & Bruford, 2006); however, the emergence of more polymorphic mtDNA markers (Tikochinski et al., 2012) opened up the possibility of using this maternally inherited molecule at finer temporal or geographical scales (Shamblin et al., 2015). Combining the mitochondrial STR haplotyping with the traditional control region haplotyping is helpful only if the latter is diverse enough (Shamblin et al., 2015). This was not carried out because the vast majority of the population (more than 97% of the samples analysed) consisted of the same control region haplotype (CM-A13), adding negligible contribution to the diversity. The application of the mtDNA STRs enabled the characterization of up to four genetically distinct nesting areas within a geographically restricted and recently colonized area. The Akamas population was the most differentiated and the least polymorphic, as it is predominantly characterized by a high frequency of the haplotype 6–8–8-4. Such low diversity might be a consequence of a founder effect from a recent colonization or the result of a bottleneck caused by a reduction of the number of nesting females (Lande, 1988). No genetic differentiation was found among all Turkish nesting sites, and therefore we combined the haplotype frequencies of all sites to form a single data set. The absence of genetic structuring using STRs supports the genetic homogeneity found among Turkish nesting areas using a ~800-bp of the control region of the mtDNA (Bagda et al., 2012), suggesting the interchange of females among nesting areas, as shown by tag–recapture methods (Sönmez, Türkecan, & Jded, 2017). Surprisingly, the same study found some structuring using microsatellites (Bagda et al., 2012), and thus the structuring among Turkish nesting sites is not yet resolved. For this reason, we strongly recommend the extensive genotyping of mtDNA STRs across the eastern Mediterranean, especially at locations along the Turkish coast and for the Syrian nesting population. Although the Israeli and the Turkish populations are geographically remote from each other they proved to be the most genetically similar, albeit still significantly differentiated. The Israeli population was remarkably the most polymorphic of all the sampled populations, despite its small population size, which was an unexpected result considering that smaller and younger populations tend to be less polymorphic because of the founder effect and the greater impact of genetic drift (Lande, 1988). This is not unprecedented, as similar results were found for the four Brazilian rookeries examined with mTDNA STR (Shamblin et al., 2015). Furthermore, loggerhead turtle nesting populations along the Israeli and the Lebanese coasts had greater polymorphism than other Mediterranean populations (Carreras et al., 2007). Marine turtle nesting populations along the eastern Mediterranean coast, such as those in Israel or Lebanon, used to be much bigger in the recent past, but have since undergone a massive decrease as a result of extensive harvesting at the beginning of the 20th century, and again during the Second World War (Levy, 2010; Sella, 1982). Considering the long generation time of marine turtles, only a few generations have passed since this population reduction. Therefore, this reported bottleneck has not yet impacted their actual mtDNA genetic diversity, and continued monitoring is recommended to track any future loss of genetic variants. In any case, the results from mtDNA STRs provide enough evidence to propose a minimum of four genetically distinct management units for this species in the Mediterranean, as generated by female philopatry, that should be taken into account in future management and conservation plans.

One of the most interesting aspects of our research was the analysis of the stranded turtles at the Israeli shores through the first STR-based MSA performed on marine turtles. The first interesting result was obtained when comparing the different life stages, as no genetic differences were found among them, meaning that the origin of the individuals is independent of the size classes. This is not unprecedented, as the Learned Migration Goal Theory predicts that adults tend to use the same foraging areas that they used as juveniles (Hays, Fossette, Katselidis, Mariani, & Schofield, 2010), and for this reason our results can be applied to the range of sizes found in this foraging area (Figure 4). Thus, most of the individuals that travelled along the Israeli coast and stranded there came from other distant populations, as expected simply by the small census size of the Israeli nesting population compared with the number of stranded animals (Levy, 2010; Levy et al., 2015).

The origin of these animals was mainly Turkey, the furthest population from the foraging area, with little to no contribution from the nearest sampled populations on Cyprus. Particle modelling suggested that individuals hatched in Mediterranean green turtle nesting areas would remain in the eastern basin (Casale & Mariani, 2014). A detailed analysis of this study revealed that Cypriot populations were predicted to use the eastern coast of the Mediterranean in a higher proportion than the Turkish populations, until the authors accounted for the population sizes at the origin from where the particles were released. These corrected estimates then revealed that up to 70% of the particles originated from Turkish nesting sites. Satellite telemetry studies in the Mediterranean have focused mainly on the Alagadi population, and have shown that a majority of nesting females migrate to the African shores after nesting, although in a few cases (two of 22) the migration route, but not the final destination, approached the Israeli coast (Stokes et al., 2015). Satellite telemetry studies do not necessarily reflect the proportion of individuals that use each of the possible migration routes, however, as a result of the low sampling size of this technique because of the implementation costs. This shortcoming has been solved by a recent study that combined satellite telemetry with stable isotope analysis to assess the use of foraging grounds of turtles from Alagadi (Bradshaw et al., 2017). Whereas

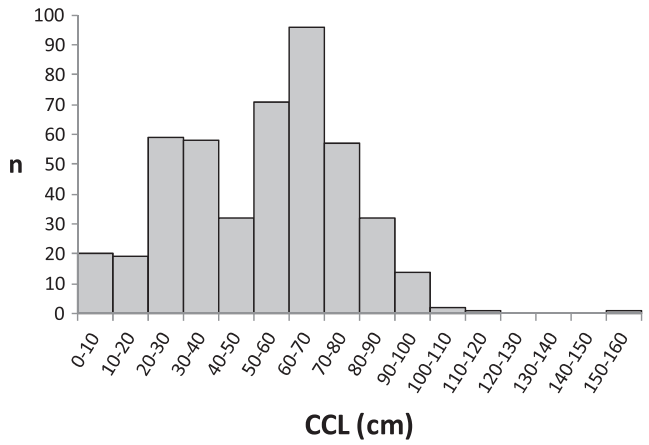

FIGURE 4 Size-class distribution of all stranded turtles recorded along the Israeli coast from 2001 to 2016. Each bar set represents the number of turtles (*n*) within each 10-cm size class, measured as curved carapace length (CCL)

telemetry tracks show the routes followed by the turtles, the extensive sampling for stable isotope analysis provided an estimate of the percentage of turtles using each one of the possible foraging grounds. Thus, turtles from Alagadi were estimated to use four different broad-scale foraging areas: Turkey–Cyprus (7%), West Libya (12%), the Gulf of Bomba (13%), and Egypt (39%), with the remaining individuals (29%) having no assigned foraging area. Considering the routes followed by turtles using each of these foraging areas, only those migrating to Egypt travel near the Israeli coast, and thus could be potentially detected among the Israeli stranded turtles. Only a few animals were tracked from Turkish rookeries (eight in total), but two of them followed the Levantine coast, heading south and as far as the Lebanese coast. Although no tracked green turtles reached the Israeli shore (Stokes et al., 2015), this does suggest that a proportion of the individuals from Turkish populations use Levantine foraging areas. Our results demonstrate that a significant proportion of Turkish individuals follow the Levantine coast and arrive in Israeli foraging areas. Additional studies incorporating stable isotopes are desirable to fully understand the habitat use of Turkish nesting turtles (Bradshaw et al., 2017). Five animals from Israel, three females and two males, were also recently tracked by telemetry showing that Israeli turtles remain in the vicinity of this area, and thus explaining why they were detected among the stranding animals (Levy et al., 2017). Furthermore, one animal has been tracked from the Syrian nesting population, and showed a reduced migratory speed coupled with foraging behaviour along Israeli coasts (Rees, Jony, Margaritoulis, & Godley, 2008). This nesting area was recently discovered in 2004 and hosts one hundred nesting females (Rees, Saad, & Jony, 2008), but unfortunately no sample could be obtained from this population for STR genotyping. The telemetry results suggest that Syria might also be contributing to the Israeli foraging area, and the presence of some orphan haplotypes suggests that the sampling of the baseline may be incomplete. For instance, the discovery of a western Atlantic orphan haplotype on the island of Poilaro revealed a new east to west transoceanic migration route for this species (Patricio et al., 2017), thus highlighting the potential impact of orphan haplotypes and the importance of good baseline sampling. In any case, this possible contribution remains to be tested by sampling this nesting population. Finally, no telemetry data have been published regarding the foraging areas used by the turtles nesting in Akamas, although our results suggest that the Israeli coast is not one of them. Considering published data and our own results, it seems that there might be a contradiction, as stable isotope analysis suggested that a significant proportion of turtles (39%) from Alagadi approach the Israeli coast on the migration to Egypt (Bradshaw et al., 2017), but this was not detected when performing an MSA (present study).

There are two non-exclusive possibilities to explain the apparent contradiction in these results. The first possibility is that the Levantine shore is a migratory corridor but not a foraging area for Cypriot turtles. Although some individuals travelled across the Israeli coast on their migration to Egypt, satellite telemetry showed that the migration speed was rapid in this area, and not indicative of foraging (Bradshaw et al., 2017). The probability of a turtle being detected by the MSA is necessarily related to the time they spent in the area and the potential for them to interact with fishing gear and other threats, so fast

migrating individuals may remain undetected. The second possibility is that we detected more individuals from the Turkish populations within the Israeli foraging area because nesting numbers are 10 times larger for these populations (Stokes et al., 2015). This is not unprecedented, as particle modelling predicted a similar effect when up to 70% of the particles that were recovered in the Levantine area originated from Turkish nesting populations, as an effect of differential population size (Casale & Mariani, 2014). Independently of the explanation of these results, it is clear that most of the individuals from the Israeli foraging area originated in Turkey, thus demonstrating the southward migration of the individuals from this area. This route may also maintain long-term connectivity as an explanation for the relative resemblance between the Israeli and the Turkish populations.

Since realizing that the Israeli green turtle population is on the verge of extinction, the Israeli Nature and Park Authority has started an initiative to increase the number of Israeli shore hatchlings released to sea. A captive breeding stock was assembled from hatchlings of two remote Israeli nests in 2001. The development of the mtDNA STR haplotyping enabled us to determine the two different haplotypes in the breeding stock. Since then, recruitment of new turtles to the captive breeding stock has been based on the haplotyping analysis of candidates. The recruitment guidelines aim to increase the variability while maintaining the representation of the original local population.

5 | CONCLUSIONS

We show that population genetic analysis is polymorphism dependent, and thus the selection of the marker used is crucial. Previous works that used less polymorphic markers found no differences among the Mediterranean populations, whereas the application of highly polymorphic mtDNA STRs showed a clear and deep genetic structuring.

Setting the geographic boundaries of different populations by their genetic composition has a major role in ecological studies and conservation. Our data already assist in setting the guidelines for the Israeli captive breeding stock, and its success in the recovery of the local population will be monitored using the same haplotyping method. A productive green turtle management programme could pave the way for similar initiatives in the region.

Our examination of migrating turtles provides another dimension to the understanding of population dynamics. We can better understand the connection and genetic exchange between populations, which is a challenging task when dealing with species that travel hundreds of kilometres in their quest for food and reproduction, a key factor of their ability to survive for more than 100 million years of extreme environmental changes around the globe.

Our study has several conservation implications for the state of the Mediterranean green turtle populations. Up to four differentiated management units have been defined for the Mediterranean green turtle populations. A substantial size decrease in any of these populations would imply the loss of a significant part of the genetic variability of the species. The Mediterranean population structuring indicates that each management unit should be conserved independently, as the recovery of one of them is not expected to favour the remaining units. Finally, the connectivity between nesting populations and distant mixed stocks, such as the Israeli coast, is crucial to detect possible sinks of individuals that may hamper the recovery of the affected populations.

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SUPPORTING INFORMATION

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