Defining conservation units with enhanced molecular tools to reveal fine scale structuring among Mediterranean green turtle rookeries

P.J. Bradshaw[a,b], A.C. Broderick[a], C. Carreras[a,c], W. Fuller[a,d], R.T.E. Snape[a], L.I. Wright[a,b], B.J. Godley[a,*]

[a] Centre for Ecology and Conservation, College of Life and Environmental Sciences, University of Exeter, Penryn Campus, Penryn TR10 9FE, UK
[b] NERC Biomolecular Analysis Facility, Department of Animal and Plant Sciences, University of Sheffield, Western Bank, Sheffield, South Yorkshire S10 2TN, UK
[c] Department of Genetics, Microbiology and Statistics and IRBio, University of Barcelona, Av.Diagonal 643, 08028 Barcelona, Spain
[d] Faculty of Veterinary Medicine, Near East University, Nicosia, Mersin 10, N. Cyprus, Turkey

ABSTRACT

Understanding the connectivity among populations is a key research priority for species of conservation concern. Genetic tools are widely used for this purpose, but the results can be limited by the resolution of the genetic markers in relation to the species and geographic scale. Here, we investigated natal philopatry in green turtles (*Chelonia mydas*) from four rookeries within close geographic proximity (~200 km) on the Mediterranean island of Cyprus. We genotyped hypervariable mtSTRs, a mtDNA control region sequence (CR) and 13 microsatellite loci to genetically characterise 479 green turtles using markers with different modes of inheritance. We demonstrated matrilineal stock structure for the first time among Mediterranean green turtle rookeries. This result contradicts previous regional assessments and supports a growing body of evidence that green turtles exhibit a more precise level of natal site fidelity than has commonly been recognised. The microsatellites detected weak male philopatry with significant stock structure among three of the six pairwise comparisons. The absence of Atlantic CR haplotypes and mtSTRs amongst these robust sample sizes reaffirms the reproductive isolation of Mediterranean green turtles and supports their status as a subpopulation. A power analysis effectively demonstrated that the mtDNA genetic markers previously employed to evaluate regional stock identity were confounded by an insufficient resolution considering the recent colonisation of this region. These findings improve the regional understanding of stock connectivity and illustrate the importance of using suitable genetic markers to define appropriate units for management and conservation.

1. Introduction

Identifying demographically independent populations is central to the management and conservation of natural populations and a key research priority for species of conservation concern (Frankham, 2010; Rees et al., 2016; Wallace et al., 2011). Molecular genetics have been used extensively to define demographically independent populations (reviewed in Waples and Gaggiotti, 2006) for management and conservation (Schwartz et al., 2007). However, populations are dynamic and alter in size and connectivity over contemporary and evolutionary time-scales.

The potential of a genetic marker to evaluate contemporary gene flow relies on its variability as this allows the detection of genetic differences in divergent lineages. The variability of a genetic marker across populations depends on marker specific rates of mutation, the number of immigrants exchanged among populations and the number

of generations over which populations have diverged (Slatkin, 1987). However, historical patterns of extinction and recolonization can mask contemporary levels of gene flow as they result in homogenising gene frequencies (Slatkin, 1987). Thus, the mutation rate of the genetic marker can reflect different temporal depths of genetic subdivision. Furthermore, the genetic diversity in newly colonised or peripheral populations is expected to be lower than in older or more centrally located populations (Eckert et al., 2008) and only highly variable markers will allow proper discrimination among intraspecific populations. Microsatellites (Selkoe and Toonen, 2006) and short tandem repeats on the mitochondrial DNA (mtSTRs) (Lunt et al., 1998; Tikochinski et al., 2012) are both highly variable as they acquire new mutations rapidly through slipped strand mispairing. However, the use of these hyper-variable markers is not free from drawbacks as they can be subject to significant levels of homoplasy (Lunt et al., 1998) and high levels of within-population heterozygosity supresses among-population
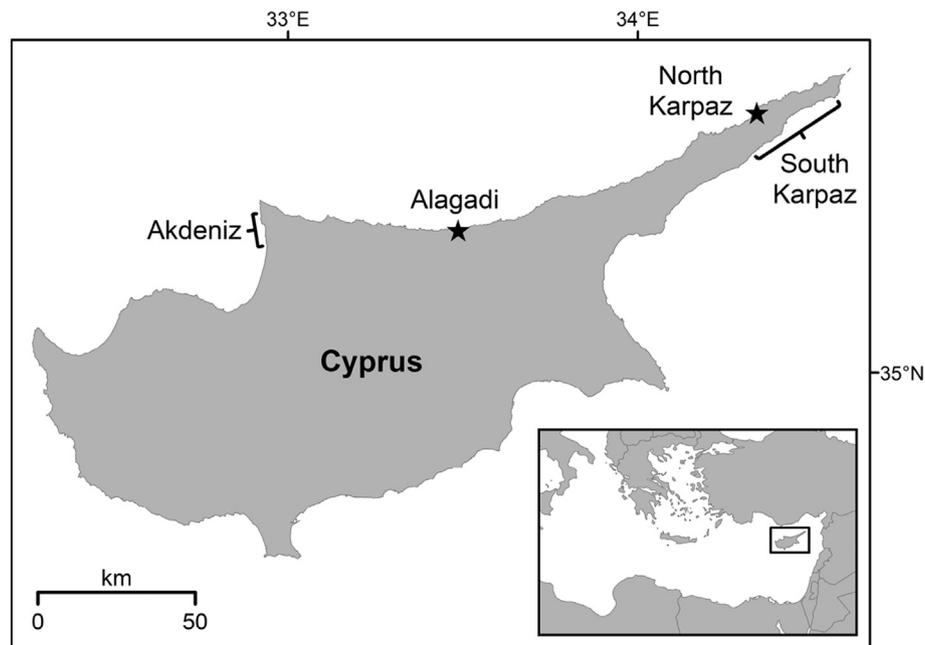
Fig. 1. The location of the Mediterranean island of Cyprus and the four green turtle rookeries sampled at Akdeniz, Alagadi, North Karpaz and South Karpaz.

differentiation as alleles become saturated (Hedrick, 1999). In summary, it is not having a marker with the most variability that is important, but to have a marker with the right amount of variability for the expected scale of differentiation. It is therefore of vital importance to select the appropriate genetic markers to define suitable management units (Karl et al., 2012; Wan et al., 2004).

Marine turtles are species of conservation concern that form distinct population segments as both sexes exhibit natal site philopatry (FitzSimmons et al., 1997; Meylan et al., 1990). Yet precise natal philopatry could be a recipe for extinction if some individuals did not stray to colonise new areas (Schroth et al., 1996). The nesting site fidelity of females defines reproductive populations, and thus units for management and conservation, as the loss of the females at a particular site will effectively result in a failure to reproduce (Bowen et al., 2005). Males on the other hand do not necessarily restrict their mating efforts to natal rookeries which is equally important for maintaining the adaptive potential of a population (Karl et al., 1992; Wright et al., 2012b). Therefore, genetic markers with different modes of inheritance are necessary to accurately evaluate the level of gene flow that connects marine turtle rookeries. To date, maternal stock structure has typically been evaluated through the use of mtDNA control region (CR) sequences due to their relatively rapid pace of evolution and non-recombining mode of inheritance (Avise, 1995). Frequency-based analyses of CR haplotypes have been very informative for defining regional management units (RMU, Wallace et al., 2010) with significant genetic differentiation typically described among rookeries separated by > 500 km (Bowen and Karl, 2007; Dethmers et al., 2006). However, mtDNA in marine turtles evolves far more slowly (~0.25% substitutions/Myr) compared to the conventional rate for other vertebrates (2%/Myr) (Avise et al., 1992) which makes it a relatively insensitive marker at finer geographic scales or among more recently divergent populations (Formia et al., 2006). More recently, some studies have used more variable genetic markers to reveal maternal genetic structure in populations that are genetically homogenous for CR haplotypes (Shamblin et al., 2012, 2015b). In contrast, the movement of males is generally inferred from microsatellites that have a higher mutation rate and a four-fold greater effective population size (Carreras et al., 2011 and refs therein). In general, less genetic structure is commonly detected using microsatellites which is attributed to male-mediated gene flow (Bowen and Karl, 2007; Karl et al., 1992). Sex-biased patterns in

gene flow is not the only factor that can produce discordance in F_{ST} levels between markers as the higher alleleic diversity of microsatellites within subpopulations, compared to mtDNA haplotype diversity, can reduce the levels of among population differentiation and increase the rate of homoplasy (reviewed in Karl et al., 2012). Thus, trying to define discrete management units based on the exact geographical specificity of natal homing in marine turtles has led to mixed conclusions dependent on the type and resolution of genetic markers (reviewed in Jensen et al., 2013; Komoroske et al., 2017).

An example of this unclear resolution was raised when evaluating the fine scale regional genetic connectivity among populations of green turtles (*Chelonia mydas*) within the Mediterranean. This region has shown an unusual pattern, as a greater genetic structure has been detected with nuclear rather than mitochondrial DNA markers, and thus the largest regional rookeries situated in Turkey and Cyprus (Stokes et al., 2015) are suggested to be a single management unit (Bagda et al., 2012; Kaska, 2000). Furthermore, the Mediterranean RMU is considered critically endangered with only 350 females nesting annually and therefore it is also of great conservation interest. (Broderick et al., 2002; Wallace et al., 2010). It is vital that the connectivity of the Mediterranean green turtle is adequately assessed as these nesting aggregations face an increasing pressure to both nesting and foraging habitats through coastal development, habitat degradation and the impact of fisheries (Casale, 2011; Casale and Margaritoulis, 2010).

Therefore, we use higher resolution mtDNA markers and a greater number of microsatellites to address the fine scale genetic connectivity among four green turtle rookeries in northern Cyprus. Critically, we evaluate the statistical power of our markers against those previously used to assess regional connectivity. These data will provide robust estimates of genetic structure in which to define appropriate units for management and conservation. Furthermore, we provide more accurate data that can be applied within mixed stock analyses of foraging areas (Bolker et al., 2007) and fisheries bycatch data (Laurent et al., 1998).

2. Methods

2.1. Sample collection and DNA extraction

Tissue biopsies were collected from 479 green turtles including 288 adults and 191 offspring from nests of unknown maternal origin during

Table 1
Genetic variability of the four green turtle rookeries.

	Nests per season	mtDNA			nDNA			
		n	h	π	k	AR	He	Ho
Akdeniz	49 (4–125)	132 (84)	0.7516 (0.0238)	$2.2E^{-03}$ $(1.4E^{-03})$	6.23	5.89	0.671	0.671
Alagadi	66 (8–236)	234	0.8066 (0.0663)	$2.9E^{-03}$ $(1.5E^{-03})$	6.69	5.77	0.662	0.671
N Karpaz	104 (38–179)	54	0.8707 (0.0727)	$3.3E^{-03}$ $(2.0E^{-03})$	6.08	5.82	0.655	0.655
S Karpaz	64 (35–107)	59 (46)	0.8676 (0.4367)	$3.1E^{-03}$ $(1.9E^{-03})$	5.31	5.20	0.645	0.652

General demographic and genetic diversity indices of sampling locations for the different sets of markers used in the present study. Abbreviations codes, n = sample sizes with amended sample sizes in parenthesis after the removal of first order relatives, h = haplotype diversity, π = nucleotide diversity, k = mean number of alleles per locus, AR = allelic richness, He = gene diversity, Ho = observed heterozygosity. HR haplotype values reported for mtDNA with CR haplotype values in parenthesis. Nests per season are indicated as a mean with range in parenthesis from Stokes et al. (2015).

the 2001–2015 breeding seasons. Biopsies were collected from four rookeries in northern Cyprus that are all located within a 200 km stretch of coast and include 59 samples of offspring collected from July until September in 2013 from several beaches within close proximity from the southern Karpaz Peninsula (hereafter termed "South Karpaz", 35°32′57.48″N; 34°23′18.60″E; Fig. 1 & Table 1), 54 samples collected from nesting females at Ronnas beach in the north of the Karpaz Peninsula during a two week period in the peak of nesting from the end of June 2014 (hereafter termed "North Karpaz", 35°35′57.67″N; 34°20′8.05″E; Fig. 1 & Table 1), 234 samples from nesting females encountered on Alagadi beach (35°19′56.17″N; 33°28′57.59″E; Fig. 1 & Table 1) where the Marine Turtle Conservation Project (MTCP) conducts an exhaustive night time monitoring programme (Stokes et al., 2014) and 132 samples from offspring collected throughout the 2013–2015 nesting seasons from several beaches within close proximity near Akdeniz (35°19′23.35″N; 32°56′11.22″E; Fig. 1 & Table 1).

Samples from adults comprised of a small skin biopsy (< 0.5 cm^2) taken from the trailing edge of the fore flipper of nesting females that were flipper tagged and had a passive integrated transponder (PIT tags) inserted to avoid pseudoreplication (Broderick et al., 2002). For clutches of unknown maternal origin, live or dead offspring were sampled post-hatching by taking a < 1 mm wide biopsy section of the marginal scute proximal to the supra-caudal scute. All biopsies were stored in 96% ethanol until genomic DNA was extracted using an ammonium acetate precipitation method (Nicholls et al., 2000).

2.2. Mitochondrial DNA

A ~800 bp fragment of the mitochondrial DNA (mtDNA) control region was amplified by polymerase chain reaction (PCR) using the primer pair LCM15382 (5′-GCTTAACCCTAAAGCATTGG-3′) and H950 (5′-GTCTCGGATTTAGGGGTTT-3′) (Abreu-Grobois et al., 2006) in a 10 μl reaction consisting of 4 μl QIAGEN Multiplex PCR Master Mix (Qiagen®; including HotStar DNA *Taq* polymerase), 3 μl ddH$_2$O, 1 μl of forward and reverse primers (5uM) and 1 μl of ~10 ng template DNA. PCR parameters included an initial hot start denaturing step at 95 °C for 15 min and then 35 cycles at 94 °C for 1 min, 52 °C for 1 min, 72 °C for 1 min 30 s and then a final extension step at 72 °C for 10 min. Furthermore, a shorter 200 bp fragment of the mtDNA control region that contains four hyper-variable dinucleotide (AT) short tandem repeats (mtSTRs) (Tikochinski et al., 2012) was amplified using primer pair CM-D-1 F (5′-AGCCCATTTACTTCTCGCCAAACCCC-3′) and CM-D-5 R (5′-GCTCCTTTTATCTGATGGGACTGTT-3′) (Tikochinski et al., 2012). We used the same reaction as for the ~800 bp mtDNA fragment but with the following PCR protocol: 95 °C for 15 min and then 35 cycles at 94 °C for 30 s, 56 °C for 30 s, 72 °C for 1 min and then a final extension step at 72 °C for 7 min.

PCR products were visualised by electrophoresis to ensure successful amplification. A total of 6 μl of the PCR amplicon was purified using 2 μl of ExoSAP-IT® (Affymetrix Inc.) and incubated as per manufacturer's instructions. Purified mtDNA amplicon was sequenced in

forward and reverse directions using the Bigdye v3.1 Cycle Sequencing Kit (Applied Biosystems™) and loaded on an ABI 3730 DNA Analyser (Applied Biosystems™). All PCR reactions were run with positive and negative controls.

All forward and reverse sequence data were aligned in Geneious v6.17 (Biomatters Ltd). Mitochondrial DNA CR haplotypes were identified using BLAST against the GenBank database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and classified according to the standardised nomenclature (http://accstr.ufl.edu/cmmtdna.html). In cases of heteroplasmy of the mtSTRs, we took the major haplotype as the consensus sequence according to peak height and aligned them manually in BioEdit v7.2.5 (Hall, 1999) to conform to the four AT short tandem repeats as described by Tikochinski et al. (2012). The mtSTRs were concatenated to the end of the mtDNA CR haplotype sequence to construct a high resolution (HR) haplotyping system using the traditional nomenclature for green turtle CR haplotypes in the Atlantic (CM-A##) followed by the four digit repeat of the mtSTRs (e.g. CM-A13.1-6_8_8_4).

2.3. Nuclear DNA (nDNA)

Samples were additionally genotyped at 13 polymorphic microsatellite loci (A6, Cm3, Or7, Cc28, Cc7E11, CcP7D04, D2, Klk314, Cm58, B103, Cc2, C102 and B123) using two multiplex reactions as per Wright et al. (2012a, b, Supplementary Information S1). Error rate in allele size scoring was assessed by repeat marker amplification of 10% of the total sample size and comparing the number of incorrect allele calls divided by the total number of alleles (Selkoe and Toonen, 2006).

2.4. Data analysis

We employed the programme Colony v2.0.5.0 (Jones and Wang, 2010) to ensure all possible effects of pseudoreplication were removed by evaluating the offspring genotypes from nests of unknown maternal origin to identify full-sibship clusters. We cross referenced the HR haplotypes and the year that the sample was collected within sibship clusters and removed all possible variants that may relate to individuals being a first degree relative (Supplementary Information S2). Temporal tests were also conducted for rookeries where samples were collected over multiple seasons and all sample years that were not significantly different were pooled for further analysis (Supplementary Information S2).

2.5. Mitochondrial DNA

Haplotype diversity (h) nucleotide diversity (π) and genetic structure were calculated using the programme Arlequin v3.5.2.3 (Excoffier and Lischer, 2010). Genetic structure was assessed through exact tests of population differentiation (default settings) and pairwise F_{ST} based on haplotype frequencies (1000 permutation tests). All multiple tests were corrected using the modified False Discovery Rate (FDR) (Narum,

Table 2
Pairwise genetic distances among the four green turtle rookeries.

	Alagadi	Akdeniz	N Karpaz	S Karpaz
Alagadi		**0.036 (0.001)**	**0.039 (0.00)**	**0.051 (0.286)**
Akdeniz	**0.003 (0.005)**		0.009 (0.00)	**0.046 (0.262)**
N Karpaz	0.003 (0.001)	**0.006 (0.010)**		0.010 **(0.162)**
S Karpaz	**0.005 (0.008)**	0.005 (0.002)	0.007 (0.008)	

Above diagonal pairwise F_{ST} values obtained from HR haplotypes (from CR haplotypes in parenthesis), below diagonal pairwise F_{ST} values based on nDNA microsatellites (D_{est} in parenthesis), Significant values are shown in bold after correcting for multiple comparisons (for a P-value < 0.05, FDR = 0.0204).

2006). Pairwise F_{ST} values were used to perform a Principal Coordinate Analysis (PCoA) using the software GeneAlEx v6.5 (Peakall and Smouse, 2012). All these analyses were conducted for both the CR and HR haplotype datasets.

2.6. Nuclear DNA

Analysis to detect deviation from Hardy Weinberg Equilibrium (HWE) and tests for linkage disequilibrium (LD) were conducted in Genepop on the web v4.2 (Raymond and Rousset, 1995; Rousset, 2008) with significant P-values from multiple tests corrected using the FDR (Narum, 2006). Evidence of null alleles was checked using Microchecker v2.2.3 software (Van Oosterhout et al., 2004). General diversity indexes (number of alleles (k) observed heterozygosity (Ho) and gene diversity (He)) were calculated for the four individual rookeries and for all rookeries combined using GenAlEx v6.5 (Peakall and Smouse, 2012, 2006). Allelic richness (AR) was calculated using the 'hierfstat' package (Goudet, 2005) as implemented in R (https://www.r-project.org/). A non-parametric Kruskall-Wallis test was used to determine differences for these genetic diversity parameters among rookeries using R.

Global F_{ST} and pairwise tests for population differentiation were conducted in GeneAlEx v6.5 (Peakall and Smouse, 2012, 2006) using frequency-based statistics including Wright's F_{ST} (1951) and Jost's estimate of differentiation (D_{est}) (Jost, 2008) with statistical significance ascertained via 999 permutation tests. Genetic differentiation based on D_{est} values were used to perform a PCoA and an isolation by distance Mantel test using the geographic distance among rookeries in kilometres following the shortest possible swimming distance along the coastline. Rookeries were checked for evidence of recent genetic bottlenecks using the one-tailed test for heterozygous excess (Cornuet and Luikart, 1996) under the two-phase model for microsatellite mutation (Roberts et al., 2004) as implemented in Bottleneck v1.2.02 (Piry et al., 1999).

To assess whether clusters of genetically similar individuals could be identified from the whole nuclear dataset, we employed two Bayesian clustering algorithms in the software programmes STRUCTURE v2.3 (Pritchard et al., 2000) and Geneland (Guillot et al., 2005) (Supplementary Information S3).

The resolution of genetic markers to detect different levels of genetic differentiation were tested using the programme POWSIM v4.1 (Ryman and Palm, 2006, Supplementary Information S4). To conduct a direct comparison of statistical power between our organelle mtDNA and nuclear DNA markers we followed the method of Larsson et al. (2009).

3. Results

All samples (n = 479) were successfully sequenced for HR haplotypes (thus including the ~800 bp CR haplotype and the mtSTRs) and genotyped across all 13 loci. The filtering process to remove putative close relatives reduced the sample sizes for South Karpaz by 13 individuals with a further 48 individuals removed from Akdeniz. No significant temporal trends in haplotype frequencies were identified for

the Alagadi or the Akdeniz rookeries and thus haplotype frequencies were pooled across years.

3.1. Mitochondrial DNA

A total of four CR haplotypes (CM-A13.1, CM-A14.1, CM-A60.1 and CM-A62.1, Supplementary Information S5) were found among the four rookeries of which two were unique to the South Karpaz. CM-A13.1 accounted for 94.3% of total samples. Control region (CR) haplotype diversity was low for Alagadi, Akdeniz and North Karpaz ($h = 0.024$–0.073; S5) but much greater within the South Karpaz rookery ($h = 0.437$; S7). Nucleotide diversity was low among all sites ($\pi = 3E^{-5}$–$5.7E^{-4}$; S5) as haplotypes only differ from CM-A13.1 through a single point mutation (Supplementary Information S6). With the inclusion of the mtSTRs, we revealed 33 HR haplotypes with the regionally common CM-A13.1 haplotype being subdivided into 28 unique haplotypes (Supplementary Information S5). Unsurprisingly, the genetic diversity in all rookeries was much greater using HR haplotypes ($h = 0.752$–0.871; Table 1) with a marginal increase in nucleotide diversity ($\pi = 2.2E^{-3}$–$3.3E^{-3}$, Table 1).

Significant stock structure was detected between the South Karpaz and the three other rookeries based on the CR marker but no stock structure was detected for any other pairwise combinations (Table 2). However, the HR haplotype marker revealed significant stock structure for four of the six pairwise comparisons that included significant differentiation between Alagadi and all other rookeries as well as between Akdeniz and the South Karpaz (Table 2). Additional stock structure was also detected between the North Karpaz and Akdeniz based on the exact test of population differentiation (Non-differentiation exact P-value = 0.01415). The PCoA based on the pairwise F_{ST} values explained 100% of the genetic variation among populations in the first two axes (Fig. 2A).

3.2. Nuclear DNA

All loci were found to be highly polymorphic ranging between 4 and 14 alleles per locus among rookeries and up to 12 alleles per locus within rookeries (Supplementary Information S7). Genotypic differences were observed at 3 allele calls of 1340 (0.22% error) with the highest error rate recorded for locus B123 (1.9%). All rookeries exhibited similar allelic richness, observed heterozygosity and gene diversity (Table 1) with no significant differences found among rookeries (Kruskall-Wallis, P > 0.76 for all tests) and no evidence of inbreeding was detected. No evidence of null alleles were found or significant departures from HWE for individual rookeries across loci or when rookeries were pooled after correcting for multiple tests (All rookeries, $\chi^2_{104} = 134.27$, P = 0.0245). Some evidence for linkage disequilibrium (LD) were found for four pairs of loci within Alagadi where close relatives could be expected due to the near complete sampling of this rookery. These loci pairs did not remain significant among rookeries with the exception for loci pair Cm3 – B123 as the P-value within Alagadi was highly significant (P < 0.001). However, no evidence for linkage was found for this loci pair within the other rookeries (P > 0.23) or in previous studies on the Alagadi rookery (Wright et al., 2012a, 2012b) and so for these reasons we assumed all loci to be independent.

Global F_{ST} averaged across loci was 0.007 (range = 0.003–0.018; Supplementary Information S8) and weak but significant genetic differentiation was found for three of the six pairwise comparisons after correcting for multiple tests (Table 2). Significant stock structure was detected between Alagadi and both the Akdeniz and South Karpaz rookeries as well as between Akdeniz and the North Karpaz with marginally non-significant structure between the North and South Karpaz (P = 0.052; Table 2). The first two axis of the PCoA explained 100% of the genetic variation among rookeries and demonstrated a north-south divide with the two rookeries on the north coast being genetically more
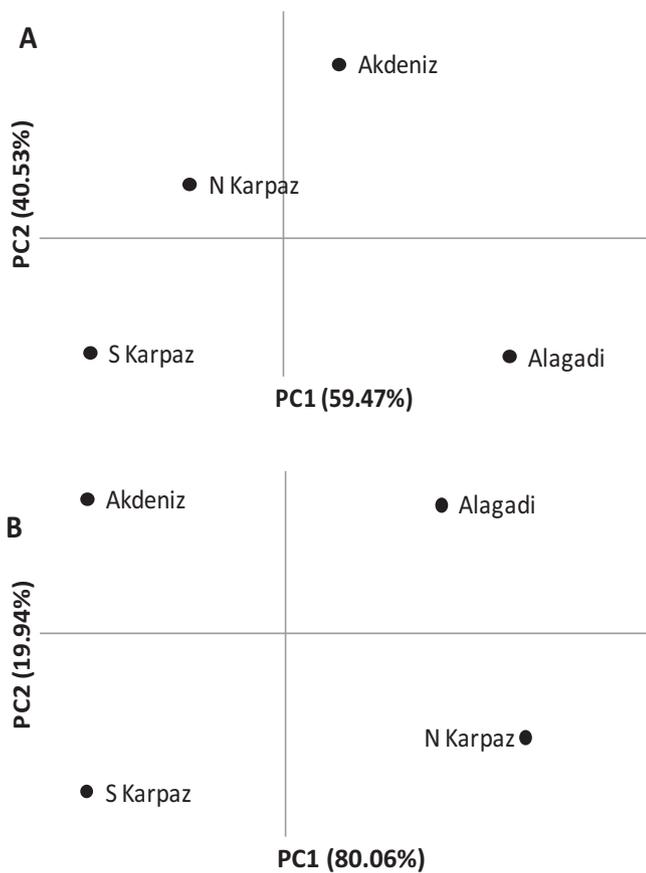
A

B

Fig. 2. PCoA using a frequency based analysis of A) F-statistics for HR haplotypes and B) D_{est} values for nDNA microsatellites. The percentage of variability explained by each principal coordinate is shown in brackets.

similar as were Akdeniz and the South Karpaz (Fig. 2B). No evidence was detected for isolation by distance with a very weak correlation between D_{est} values and geographic distances (Mantel test, $R^2 = 0.0115$, P = 0.323).

No genetic structure was inferred from the spatial and non-spatial Bayesian analyses with all individuals forming a single genetically similar cluster based on the approximation of the posterior probability for the STRUCTURE analysis (Supplementary Information S9) and the highest log-likelihood of the posterior probability in Geneland. However, Evanno's ΔK from the STRUCTURE analysis (Evanno et al., 2005) suggested two or four genetically similar clusters in turn (Supplementary Information S10) which may relate to genetic sub-structuring as they failed to show any geographic coherence with low individual admixture coefficients within any single cluster. No evidence was found for recent genetic bottlenecks for any rookery under the two-phase model (one-tailed test for heterozygote excess P > 0.07 in all cases).

3.3. Analysis of statistical power for genetic markers to detect population structure

The POWSIM analysis revealed that the CR haplotype marker had an insufficient statistical power to detect significant stock structure considering the levels of genetic differentiation found in this study. As predicted, the statistical power was greatly increased using the HR system and an acceptable Type 1 error rate was maintained across the range of the F_{ST} values (Fig. 3). The microsatellite array used in this study exhibited the greatest statistical power among markers but there was a slight elevation in the Type 1 error rate.

4. Discussion

This study effectively demonstrates the need to employ genetic markers with the appropriate level of variability for the study species and the temporal and geographic context of the studied populations. For the first time, through the use of higher resolution mtDNA markers, we reveal matrilineal stock structure amongst Mediterranean green turtle rookeries. The structure detected among these four rookeries that are geographically separated by 60–200 km suggest that natal site fidelity within the Mediterranean is much greater than previously suggested (Bagda et al., 2012) with serious implications for the management and conservation of green turtles. Evidence for male philopatry was detected, but F_{ST} values were an order of magnitude lower than those found with the HR haplotypes which suggests some male-mediated gene flow does exist. The absence of shared CR haplotypes and mtSTRs provides greater evidence for the designation of the Mediterranean as an independent Regional Management Unit (RMU) from conspecific rookeries within the Atlantic.

In relation to other long-lived marine vertebrates (e.g. Hoelzel et al., 1991), marine turtle mtDNA control region sequences accumulate new mutations at a very slow rate (0.2×10^{-8} My^{-1} (Encalada et al., 1996)). Therefore in areas such as the Mediterranean that are likely to have been colonised within the last 10,000 yrs. (Bowen et al., 1992; Clusa et al., 2013), the mtDNA CR marker is unlikely to have acquired a sufficient number of new mutations to detect recently divergent populations. However, the Mediterranean case study is unlikely to be an isolated situation. Many marine turtle rookeries will have experienced a series of colonisation and extirpation events due to sea level fluctuations from glacial and interglacial cycles (Naro-Maciel et al., 2014; Reece et al., 2005) as well as a variety of anthropogenic threats (Broderick et al., 2006; McClenachan et al., 2006), environmental variability and nesting beach dynamics. Hence, the CR marker is more likely to reflect historical events as opposed to contemporary levels of gene flow (Formia et al., 2006). The power analysis clearly demonstrated that the CR marker is an insensitive marker with which to reject panmixia amongst recently colonised populations and therefore it is likely to have underestimated stock structure among other globally distributed regional rookeries (e.g. Shamblin et al., 2012, 2015a, 2015b). The misidentification of appropriate management units raises important conservation implications as contemporary stock structure, as defined by the CR haplotypes, may in fact have grouped demographically independent breeding stocks. Incorrectly specifying conservation management units could lead to the loss of discrete breeding stocks if the reproductive females and nesting habitat are not afforded an adequate level of protection as population growth rates depend more on local birth and death rates than on immigration. Therefore green turtle rookeries might be very slow to re-establish once extirpated as the geographic extent of the genetic structure detected here suggests a high level of natal site fidelity and challenges previous conceptions for the geographic scale of independent breeding stocks elsewhere (Dethmers et al., 2006). Therefore, the addition of mtSTRs to the CR sequence to define HR haplotypes might be a more appropriate marker in which to assess contemporary stock structure and they can also yield more accurate assignments of individuals to natal rookeries from mixed stock analyses.

The results from our mtSTR analysis supported the designation of the Mediterranean green turtle RMU through the absence of shared mtSTRs with Atlantic rookeries (Shamblin et al., 2015a, 2015b). So far, all 44 variants of the mtSTRs discovered within the Mediterranean appear near-endemic (Tikochinski et al., 2012; this study) which is further corroborated by the distinct absence of any Atlantic CR haplotypes, despite our robust sample sizes. Although the Mediterranean was colonised by green turtles from the Atlantic (Naro-Maciel et al., 2014) and there is no physical barrier to dispersal (although see Revelles et al. (2007)) only two CR haplotypes (CM-A13.1 and CM-A27.1) are known to co-occur in both regions (Encalada et al., 1996; Kaska, 2000;
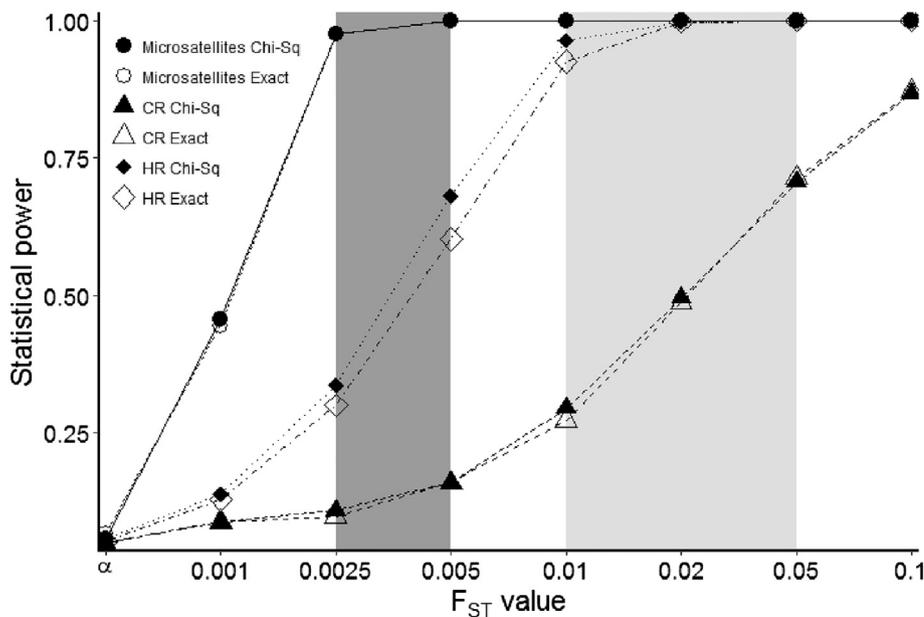
Fig. 3. Statistical power of the genetic markers to detect divergence at different levels of F_{ST} for mtDNA CR and HR markers and the 13 microsatellites for chi-square and exact tests of population differentiation. Shaded areas indicate the range of F_{ST} values found within this study, dark grey = microsatellites, light grey = mtDNA HR haplotypes. Power is expressed as the proportion of significant values ($P < 0.05$) using empirical sample sizes.

Shamblin et al., 2015a) suggesting that gene flow has been limited over an ecological time-scale.

Assumptions of sex-biased gene flow were confirmed among these Mediterranean rookeries as microsatellite markers revealed a lower, albeit significant, genetic structure among three of the six pairwise comparisons. The F_{ST} values among rookery pairs were generally an order of magnitude lower than those found for mtDNA and this can be expected when comparing genetic markers with different modes of inheritance in addition to differences in marker evolution (reviewed in Karl et al., 2012). Furthermore, male marine turtles are expected to exhibit a lower level of philopatry as they do not need to find a suitable nesting site, only to find reproductive mates. However, male turtles still exhibit philopatry (FitzSimmons et al., 1997) and significant genetic structuring was previously detected amongst green turtle rookeries in the Mediterranean (Bagda et al., 2012), as in the present study. In general, weak but significant genetic structure is increasingly being revealed within studies that employ a greater numbers of microsatellites (e.g. Carreras et al., 2011; Dutton et al., 2013; Naro-Maciel et al., 2014).

5. Conservation implications

With the advancements in molecular genetics over the last decade, it is time for new regional assessments of connectivity among marine turtle rookeries that are currently considered to be genetically homogenous with higher resolution mtDNA markers, a greater number of microsatellites or single nucleotide polymorphisms (SNPs). We advise that the green turtle rookeries in northern Cyprus, and most likely the wider Mediterranean, should be considered as separate units for conservation and management to avoid the loss of independent breeding stocks.

Acknowledgements

Appendix A. Data accessibility

The 'mainparams' file for the STRUCTURE analysis and a list of micro- satellite genotypes with CR and HR haplotypes of all individuals (filtered 500 and unfiltered), including individual ID, sampling location and cohort information as described in Table 1, is available through Dryad Digital Repository at https://datadryad.org/resource/doi:10.5061/dryad.7db01.

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