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Molecular techniques reveal cryptic life history and demographic processes of a critically endangered marine turtle

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ABSTRACT

The concept of 'effective population size' (N_e), which quantifies how quickly a population will lose genetic variability, is one of the most important contributions of theoretical evolutionary biology to practical conservation management. N_e is often much lower than actual population size: how much so depends on key life history and demographic parameters, such as mating systems and population connectivity, that often remain unknown for species of conservation concern. Molecular techniques allow the indirect study of these parameters, as well as the estimation of current and historical N_e. Here, we use genotyping to assess the genetic health of an important population of the critically endangered hawksbill turtle (*Eretmochelys imbricata*), a slow-to-mature, difficult-to-observe species with a long history of severe overhunting. Our results were surprisingly positive: we found that the study population, located in the Republic of Seychelles, Indian Ocean, has a relatively large N_e, estimated to exceed 1000, and showed no evidence of a recent reduction in N_e (i.e. no genetic bottleneck). Furthermore, molecular inferences suggest the species' mating system is conducive to maintaining a large N_e, with a relatively large and widely distributed male population promoting considerable gene flow amongst nesting sites across the Seychelles area. This may also be reinforced by the movement of females between nesting sites. Our study underlines how molecular techniques can help to inform conservation biology. In this case our results suggest that this important hawksbill population is starting from a relatively strong position as it faces new challenges, such as global climate change.

1. Introduction

Small populations lose genetic variation much more rapidly than large populations, as they are more susceptible to inbreeding and more strongly affected by genetic drift (Wright, 1931). Importantly, almost all populations will lose genetic variation more quickly than expected from their census population size N, due to factors that include variation between individuals in reproductive success, fluctuations in population size, unequal sex ratios, and population structure. This greater rate of loss is quantified as the population's effective size N_e (Wright, 1931), which is often substantially lower than N (Frankham, 1995; Hartl, 1988). Given that low genetic diversity increases the risk of population extinction and may reduce adaptability to future environmental change (Frankham et al., 1999; Franklin and Frankham, 1998), N_e and its implications for genetic diversity are important considerations in the management of species of conservation concern (Frankham et al., 2002).

Amongst conservation-priority species, demography and life history are often not well known enough for their impacts on N_e to be assessed, which restricts the potential for adjusting management plans to help solve specific conservation problems (Hare et al., 2011; Palstra and Ruzzante, 2008). In such situations, molecular techniques are essential tools, allowing mating systems to be assessed, migration and dispersal patterns to be explored, and inbreeding and genetic diversity to be quantified. Of particular value to conservation managers is the utility of molecular methods for inferring connectivity/structure amongst populations, to identify and measure the breeding contributions of unseen individuals, to derive estimates of N_e directly from molecular data, and to infer past changes in N_e such as population bottlenecks (e.g. Frankham et al., 2002; Piry et al., 1999; Waples, 1989).

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Population declines driven by overhunting, habitat loss, and other anthropogenic factors have made marine turtles a global conservation priority (Wallace et al., 2011). However, little is known about N_e in most populations of these taxa, or about how N_e might relate to census counts. This makes it difficult to quantify loss of genetic variation, or assess how low levels of variation may slow population recovery and reduce adaptability to future perturbations such as global climate change (see Hawkes et al., 2009; Wright et al., 2012b). To estimate N_e and adjust conservation management accordingly, we require more information about specific key life history and demographic parameters than is currently available for many marine turtle populations. For example, male reproductive skew is a key parameter influencing effective population size, with N_e being larger the more evenly reproduction is distributed amongst males within the population (Hartl, 1988). In marine turtles, the vast majority of paternity studies have focused on data from a single nesting season (but see Lasala et al., 2013; Wright et al., 2012a), but accurate assessment of skew in such long-lived species requires assessing paternity across years. If the same set of males sires the offspring of a given nesting site across years, skew will be higher and N_e lower than if the number/local turnover of males is greater. Furthermore, the ability to estimate N_e directly from molecular data (e.g. Wang, 2009; Waples, 1989), and to use these data to infer past changes to N_e (e.g. Garza and Williamson, 2001; Piry et al., 1999), have rarely been applied to marine turtles (but see Rivalan et al., 2006; Theissinger et al., 2009).

The hawksbill turtle (*Eretmochelys imbricata*) occurs throughout the world's tropical oceans, and is IUCN-listed as critically endangered following substantial population declines driven by anthropogenic factors (Mortimer and Donnelly, 2008). Many aspects of the hawksbill's life history are poorly known, and most published genetic work involves hawksbill populations in the Caribbean (Blumenthal et al., 2009; Bowen et al., 2007). In the Indo-Pacific, little is known about the distribution of genetic variation beyond the existence of broad-scale structure between several major rookeries (Vargas et al., 2013), and gene flow between both juveniles and nesting females of two of the region's most important populations, those of Seychelles and Chagos (Mortimer and Broderick, 1999; Sheppard et al., 2012). However, a study of mating systems based on one year's data from hawksbills in the Republic of Seychelles indicated that the number of males in this population was large, based on evidence that the majority of females were fertilised by a single male each but that no male fertilised more than one female (Phillips et al., 2013). Here, we use a four-year dataset from the same population to quantify N_e and compare it to census data, to test for changes in N_e in the recent past that might indicate genetic effects of population declines, and to assess key processes affecting N_e, such as dispersal and between-year patterns of parentage. Using samples collected from nesting beaches spanning several hundred kilometres across Seychelles, we also assess population genetic structure and consider the implications of our results for N_e and for ongoing hawksbill conservation management in the region. Our results help us move towards a fuller understanding of demographic and life history parameters in a species that is inherently difficult to study, and reiterate the value of molecular techniques to conservation biologists.

2. Methods

2.1. Field sampling

Tissue samples were collected from female hawksbills and ca 20 hatchlings per nest on Cousine Island (04°21′S, 55°38′E), Republic of Seychelles, over four nesting seasons (Sep–Apr) spanning Sep 2007–Apr 2011. For a full field protocol as used on Cousine, see Phillips et al. (2013). Over the first three years, sampling of females and nests was near exhaustive. In 2010/11, samples were only collected from previously unsampled adult females, and from hatchlings from the nests of females observed on Cousine in any of the three previous seasons of the study.

For analysis of population structure, tissue samples were collected in the 2010/11 and 2011/12 seasons from females nesting on additional islands across the Seychelles (Fig. 1): in the Granitic Seychelles (the region that includes Cousine; Fig. 1C), from Frégate (04°35′S, 55°57′E) and North Islands (04°24′S, 55°15′E); and in the Amirantes (the outer coralline islands; Fig. 1B), from D'Arros/St. Joseph (05°25′S, 53°19′E), Desroches (05°42′S, 53°40′E), and Alphonse/St. François (07°04′S, 52°44′E). Additionally, a small number of juvenile hawksbills were hand-captured and sampled in the waters around Aldabra Atoll (09°26′S, 46°23′E). These samples were collected by removing a small section of tissue from the trailing edge of a flipper with a sterile scalpel, ideally during nesting for adult females. On captive turtles, no long-term harm has been detected from comparable tissue sampling (Bjorndal et al., 2010).

2.2. Molecular analysis

Following DNA extraction (ammonium acetate method; Nicholls et al., 2000), individuals were genotyped at 32 microsatellite loci in three polymerase chain reaction multiplexes, as described in Phillips et al. (2013). An individual's genotype for a given multiplex was not used in downstream analyses if more than four loci (out of the 10–11 loci) from that multiplex failed to amplify, and individuals were removed entirely if two multiplexes were discounted, or if more than ten loci failed in total. Where possible, we genotyped at least 20 offspring per female from the 2007/08 and 2008/09 seasons. Time and cost constraints meant that we were unable to do this for 2009/10: instead, we genotyped 3 offspring from every female, and an additional 10–12 offspring from a subsample of 20 families.

2.3. Parentage assessment and reconstruction of male genotypes

After checking estimated null allele frequencies (CERVUS 3.0; Marshall et al., 1998) and assumptions of Hardy–Weinberg and linkage equilibria (GENEPOP 4.2; Raymond and Rousset, 1995; Rousset, 2008), parentage analysis was conducted on the entire Cousine dataset using COLONY 2.0 (Wang and Santure, 2009). This programme uses a maximum likelihood method to group offspring into full- and half-sib clusters, assign parentage, and reconstruct the genotypes of unsampled parents. COLONY was provided with per-locus estimates of genotyping error (Phillips et al., 2013) and allowed to update allele frequencies during the analysis. We ran the programme with three different random number seeds, with each run of 'medium' length and 'medium' precision. COLONY reconstructs the genotypes of unsampled parents on a locus-by-locus basis and provides a confidence value for each reconstruction. When assembling these into multilocus male genotypes, we only incorporated single-locus genotypes with confidence of ≥ 0.90, and only used multilocus genotypes in downstream analyses if they contained $\geq 29/32$ loci and were reconstructed from ≥ 10 offspring (see Phillips et al., 2013).

The programme COANCESTRY 1.0 (Wang, 2011) was used to screen the data for related adults prior to running any subsequent analyses, as some population genetics and N_e estimation methods can be adversely affected by the presence of close kin. Allele frequencies for use in COANCESTRY were obtained from three runs of COLONY on the entire dataset, with all adult females as candidate mothers and the Aldabra juveniles as offspring.

2.4. Population structure

Pairwise F_{ST} values and absolute number of migrants exchanged (M; Slatkin, 1991) were computed between all population pairs in ARLEQUIN 3.5 (Excoffier et al., 2005). The inbreeding coefficient F_{IS} (Wright, 1965) was also computed for each population. Male genotypes

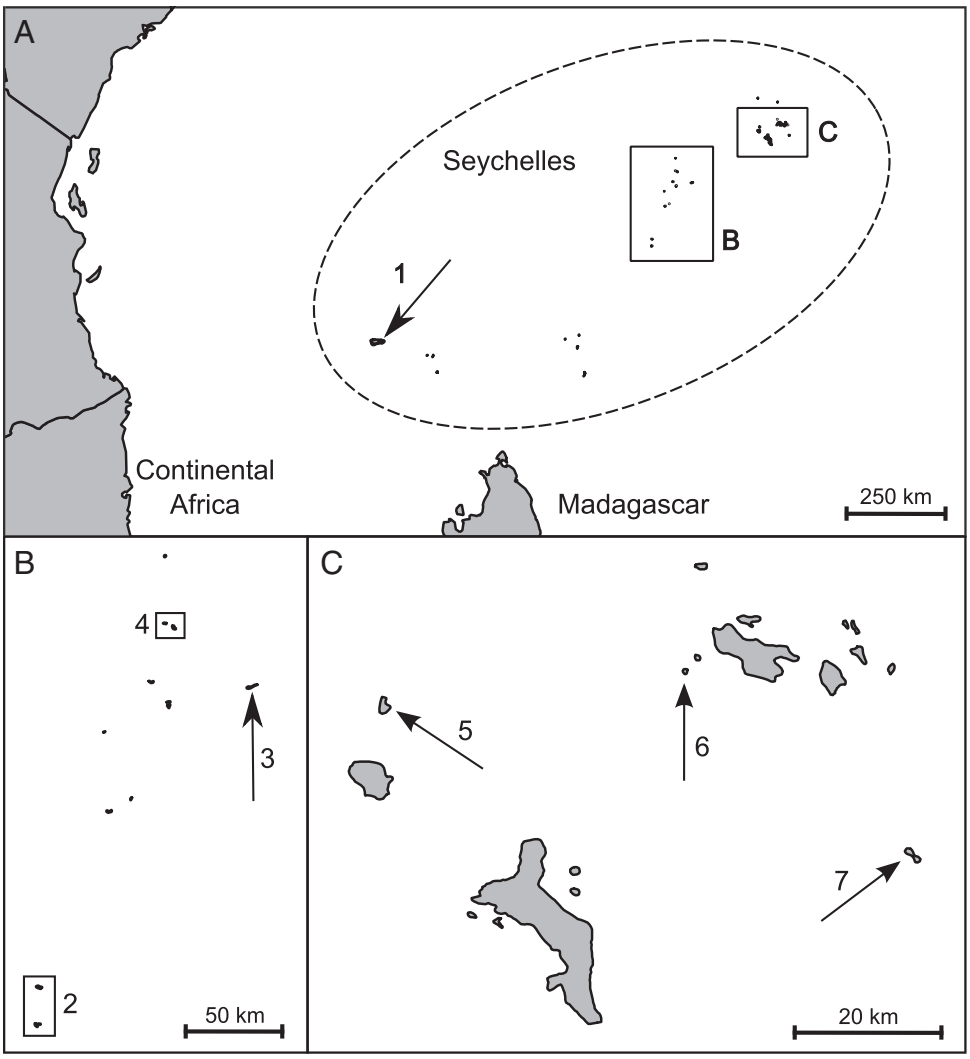

Fig. 1. Map of the study region, highlighting islands from which samples were collected. A: location of Seychelles archipelago (circled region), with close-ups of the Amirantes (B) and Granitic (C) groups. 1 = Aldabra; 2 = Alphonse and St. François; 3 = Desroches; 4 = D'Arros and St. Joseph; 5 = North Island; 6 = Cousine Island; and 7 = Frégate Island.

inferred from Cousine nests were treated as a separate population in case males and females were not from the same genetic stock (FitzSimmons et al., 1997b; Lee et al., 2007). F_{ST} was also calculated after grouping the islands by region (Granitics v. Amirantes). For an additional regional analysis, we used ARLEQUIN to perform a nested analysis of molecular variance (AMOVA), with islands nested within their respective regions. We excluded Aldabra juveniles from these 'regional' analyses because juveniles on feeding grounds may come from multiple rookeries (e.g. Blumenthal et al., 2009; Bowen et al., 2007). We also excluded Cousine-inferred males (see above). To test for alternative, potentially cryptic population structure arrangements, we ran the data through the programme STRUCTURE 2.3 (Pritchard et al., 2000), which uses a Bayesian method to cluster samples into groups that minimise deviation from Hardy–Weinberg and linkage equilibria. We ran the programme with a burn-in period of 50,000 steps and a sampling period of 100,000, using an admixture model with correlated allele frequencies. We tested for populations in the range $k = 1$–10 (we chose ten as the upper limit in case any of the eight initial groupings contained substructure, such as if males or juveniles were sourced from multiple stocks), and selected the best value of k for our data by taking the mean of the 'ln probability of data' output statistic across ten replicate runs of the analysis, and then using this to calculate posterior probabilities for each tested value of k as described in the STRUCTURE manual (Pritchard et al., 2000). If $k = 1$ had the highest posterior probability, we accepted this and reported the P-value; otherwise, we applied the method of Evanno et al. (2005) to select the best value of k. We repeated this analysis using three different location priors: no prior, island, and region.

2.5. Sex biased dispersal

To test for sex-biased dispersal, we used the assignment index method of Mossman and Waser (1999), implemented in GENALEX 6 (Peakall and Smouse, 2006). The sex with the lower index value is the more dispersive. Note that this method required that we only used genotypes with no missing data. We also undertook a relatedness-based test by using COANCESTRY to compare mean relatedness r (Queller and Goodnight, 1989) of female–female and male–male dyads using 10,000 bootstraps. A significant result would suggest that the sex with the lower average relatedness is the greater disperser (as dispersal increases, one expects to find fewer relatives within a given area). To aid interpretation of the relatedness test, we ranked all dyads by r, including male–female dyads, and calculated the proportions of each dyad class above increasing thresholds of r. If one sex is less dispersive than the other, one would predict that that sex should account for a disproportionately large share of 'higher relatedness' dyads (e.g. half-sibs ($r \approx 0.25$), full sibs ($r \approx 0.5$), and equivalents).

2.6. Estimates of effective population size

We applied four one-sample N_e estimation methods: the heterozygosity excess method (Pudovkin et al., 1996; Robertson, 1965), implemented in COLONY; the linkage disequilibrium method (Hill, 1981), implemented in LDNE 1.31 (Waples and Do, 2008); the sibship method (Wang, 2009), implemented by running adults through COLONY as 'offspring' (three runs, no prior allele frequencies); and approximate Bayesian computation (ABC), implemented in DIY ABC 1.0 (prior N_e range 100–10,000; Cornuet et al., 2008). DIY ABC was run three times, each using a different random subsample of the Cousine dataset to reduce computation times (40 females, 40 males). Each run generated 1,000,000 simulated datasets using an N_e prior range of 100–10,000. We also tried the ABC programme ONeSAMP 1.2 (Tallmon et al., 2008), but found its analysis return times for our dataset to be too long and unpredictable. We did not apply any multi-sample N_e estimation methods (e.g. Waples and Do, 2010) because our sampling period of four years is substantially lower than any marine turtle generation time (35 years for hawksbills; e.g. Meylan and Donnelly, 1999; see also Miller, 1997; Spotila, 2004), and because it is not possible to age marine turtles accurately in the field. We add the cautionary note that our study system, like most wild systems, is likely to violate some of the assumptions that underlie N_e estimation. Most notably, a sample of adult marine turtles will not represent a single cohort, and generations are likely to overlap. This is discussed later.

2.7. Bottleneck testing

We applied three methods to test for genetic evidence of past changes in effective population size. First, the programme BOTTLENECK (Piry et al., 1999), which compares a sample's heterozygosity (H_e) at each locus with that expected under mutation-drift equilibrium (H_{eq}). Heterozygosity excess ($H_e > H_{eq}$) suggests a population contraction (i.e. a bottleneck), whereas a heterozygosity deficit suggests a population expansion (Cornuet and Luikart, 1996). We used a two-phase model with parameters recommended by the programme's authors (non-stepwise = 5%, variance = 12). We assume that the population is either closed, or that the frequency with which occasional immigrants introduce new alleles does not cause significant deviations from the 'non-stepwise' component of the mutation model. BOTTLENECK does not estimate the timing or magnitude of any inferred change in N_e. Second, we used a modification of the DIY ABC scenario described in Section 2.6 to include a 'vary N_e' event at an unspecified time t in the population's history. Thus t (prior range 1–500 generations) and historical N_e (prior range 100–10,000) were included as parameters for which to estimate posterior probabilities. DIY ABC also allows for comparisons between scenarios using posterior probabilities, enabling us to assess whether the 'vary N_e' scenario had greater support than the constant N_e scenario described above. Third, we took advantage of DIY ABC's significance testing of the Garza–Williamson index, calculated for each microsatellite locus as allelic richness/locus size range (Garza and Williamson, 2001). During a population reduction, richness declines more rapidly than size range, and so low values of the index can indicate a bottleneck.

2.8. Estimating male population size

Too few males were 'genetically re-sighted' to allow mark-recapture estimation of population size according to standard methods (Greenwood and Robinson, 2006). Instead, to provide indicative figures, we conducted simulated sampling from a range of male population sizes (N_{mal}) in R (R Development Core Team, 2008). For a given N_{mal}, four samples were drawn, of sizes corresponding to the number of inferred males in each year of our study. We then calculated the modal number of re-sightings for each N_{mal} value, based on 10,000 replicates per value and compared this with the number of actual re-sightings observed within and across years in our wild sample. This basic model assumes a closed population, no mortality or recruitment, and no between-individual or temporal variation in re-sighting probability. This last assumption implies that mating is random, and that a male is as likely to be re-sighted within a year as he is between years. We also ran variations of the model to restrict male re-sightings to between years, to make some males only breed biennially, and to allow some males to be more successful in obtaining paternity than other males.

3. Results

3.1. Cousine Island parentage patterns

We genotyped 180 adult females and 3162 hatchlings (249 nests), and from this were able to reconstruct 91 male genotypes meeting our confidence criteria. All three runs of COLONY converged on the same result. Genotyped hatchlings represented the progeny of 128 females (1.82 ± 1.18 nests per female (mean ± SD), max. = 6), of which 12 females were unsampled. Four females per year in each of the first three years produced cohorts of offspring fathered by multiple males within a season. Paternity patterns were consistent with those described from a single season's data in Phillips et al. (2013), with all cases of mixed paternity attributable to two males per female, and with both males in all cases fathering offspring across their female's consecutive clutches (for a full breakdown of paternity patterns, see Tables S1 and S2). With respect to adult turtles present in multiple years, 12 sampled females laid eggs in two seasons, and four males were inferred to have fathered offspring in two seasons (Table S1). One male–female pair was observed to have reproduced together in two years (Y1 and Y3). All other re-sighted individuals had new partners in each year. For two five-member full-sib clusters, identified by COANCESTRY amongst the adult females sampled from Cousine, only one member per cluster, selected at random, was retained in downstream analyses.

3.2. Population genetics

Sample sizes from the seven sampled islands are given in Table 1. No population (island or region) had an F_{IS} significantly different from zero. Between-island pairwise F_{ST} statistics and estimates of number of migrants per generations are summarised in Table S3. Mean overall F_{ST} was 0.001 ± 0.006 (SD), with a maximum pairwise value of 0.014 between Desroches and Alphonse/St. François. Three F_{ST} values were significant at the $P < 0.05$ level and one at $P < 0.01$, all involving comparisons with Desroches. However, none of these values remained significant following correction for multiple comparisons (q-values; Storey, 2002). At the region level, F_{ST} between the Granitics and Amirantes was 0.001 ($P = 0.14$), implying a high per-generation migration rate between the groups (M = 648.0). Nested AMOVA reiterated the results of both island and region F_{ST} testing, with 99.9% of genetic variation accounted for at the 'within-island' level. Ten runs of STRUCTURE with no location priors all returned $k = 1$ with posterior probability $P > 0.99$. The same was true with island as a location prior. With region as a prior, results of individual runs were more varied, but favoured $k = 1$ with $P = 0.96$. No interpretive differences were made by re-running F_{ST} or STRUCTURE analyses using only a random subset of 40 Cousine females and 40 Cousine males in with all the other islands' samples, to avoid bias arising from having a much larger set of samples from Cousine (data not shown).

3.3. Sex-biased dispersal

Four females and 15 males were excluded from assignment index analysis because of incomplete genotypes. Mean index values were −0.48 ± 2.86 (SD; n = 76) and 0.22 ± 3.04 (n = 166) for males and

Table 1
Sample sizes and summary statistics for samples collected from seven islands across Seychelles, and for males inferred from parentage analysis on Cousine Island hatchlings. Summaries are also given for regional island groupings (Amirantes: Alphonse/St. François, D'Arros/St. Joseph, and Desroches; Granitics: Frégate, North, and Cousine females).

Population	n	#Alleles	Private alleles	H_O	H_E	F_{IS}
Aldabra (juveniles)	14	5.8 (2.5)	2	0.66 (0.18)	0.69 (0.16)	0.04 (0.21)
Alphonse/St. François	10	5.6 (2.1)	3	0.71 (0.17)	0.71 (0.14)	0.00 (0.18)
D'Arros/St. Joseph	38	7.7 (3.5)	3	0.69 (0.15)	0.70 (0.14)	0.01 (0.09)
Desroches	13	5.6 (2.0)	1	0.68 (0.18)	0.67 (0.15)	−0.01 (0.14)
Frégate	32	7.4 (3.3)	3	0.70 (0.18)	0.70 (0.15)	0.00 (0.10)
North	9	5.2 (2.1)	0	0.69 (0.23)	0.69 (0.18)	0.00 (0.19)
Cousine (females)	180	9.3 (4.8)	10	0.69 (0.13)	0.69 (0.13)	−0.01 (0.06)
Cousine (males)	91	8.8 (4.4)	4	0.70 (0.13)	0.69 (0.13)	−0.01 (0.06)
Amirantes (females)	62	8.3 (3.7)	15	0.69 (0.14)	0.70 (0.13)	0.01 (0.08)
Granitics (females)	221	9.5 (5.0)	55	0.69 (0.14)	0.69 (0.14)	−0.01 (0.05)

n = number of genotyped individuals; H_O = observed heterozygosity; H_E = heterozygosity expected under Hardy–Weinberg Equilibrium; F_{IS} = average level of individual inbreeding relative to the subpopulation.

females respectively. This difference was not significant (Wilcoxon rank sum test: W = 5504, $P = 0.11$). However, males and females differed significantly in their distributions of the index (Kolmogorov–Smirnov test: D = 0.20, $P = 0.03$), possibly due to higher kurtosis in males (1.79 v. 0.03).

Mean relatedness amongst Cousine Island females was significantly higher than mean relatedness amongst males, although the effect size of 0.008 was small (females = 0.000 ± 0.108, n = 14,365; males = −0.008 ± 0.105, n = 4095; $P < 0.001$). The effect remained when the test was re-run using random subsets of female–female dyads of balanced sample size with the males (n = 4095; three subsets: effect sizes = 0.006–0.009, $P < 0.001$–0.005). The proportional representation of female–female dyads increased rapidly as r increased above 0.20, accounting for 47.0% of all dyads with $r \geq 0.20$ (n = 1041), but 87.1% of dyads with $r \geq 0.45$ (n = 31; Fig. 2). This indicates that high-similarity pairs of individuals are likely to consist of two females.

3.4. Effective population size

The heterozygosity excess method returned 95% confidence intervals ranging from zero to infinity, which may indicate our population is too large for precise application of this method (Luikart and Cornuet, 1999). The mean N_e estimate of three runs of the sibship method was 485 (95% CI = 418–562). The linkage disequilibrium method estimated N_e at 2407 (1578–4872). Results from application of these methods to specific data subsets are given in Table 2. Three runs of DIY ABC converged on similar solutions to each other, with median N_e of 1020–1150 (178–8743).

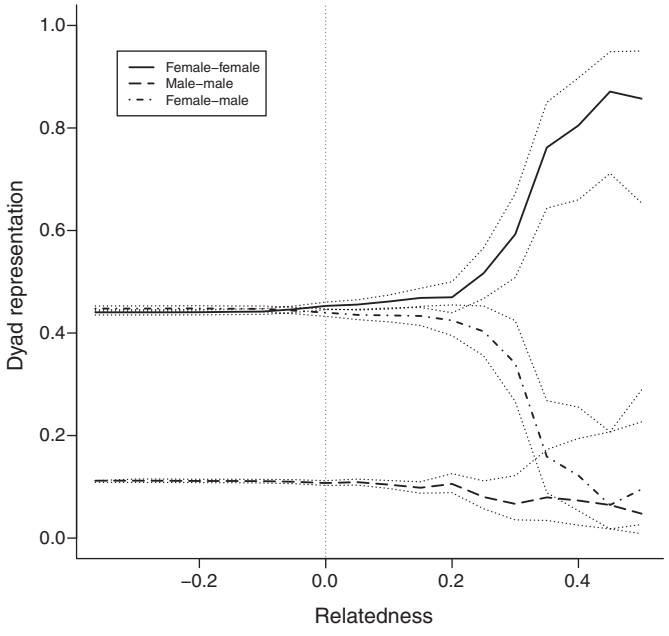

Fig. 2. Female–female dyads make up an increasingly large proportion of dyads with higher relatedness (r) values. Female–female dyads account for 44% (16 110/36 585) of all dyads, but 87% (27/31) of dyads with $r \geq 0.45$. Dotted lines indicate 95% CIs.

3.5. Tests for bottlenecks

BOTTLENECK (two-phase mutation model) returned a highly significant heterozygosity deficiency ($P = 0.001$). Re-running the analyses using subsamples of the Cousine data, or on specific data subsets (e.g. Amirantes females, non-Cousine Granitic females) made no interpretative differences (data not shown). Three runs of DIY ABC allowing a historical change in N_e converged on a scenario of population increase, summarised in Table 3. This 'vary N_e' scenario returned substantially higher estimates of current N_e than the 'constant N_e' scenario summarised above (4670–4770 v. 2060–2220; Table 3), although 95% confidence intervals showed a large overlap (406–9747 v. 178–8743). Comparing the 'vary N_e' and 'constant N_e' scenarios, DIY ABC's logistic regression estimate of posterior probabilities did not favour one model over the other: 'vary' = 0.49 (95% CI = 0.44–0.54) v. 'constant' = 0.51 (0.46–0.56). Significance testing of the Garza–Williamson (GW) index in DIY ABC indicated that the observed index values were on the edge of significance (GW = 0.76–0.77; 'constant N_e': $P = 0.03$–0.06; 'vary N_e': $P = 0.05$–0.06). Note that the whole dataset (i.e. including all Cousine samples) had GW = 0.84 ± 0.19 (SD).

3.6. Number of males in the overall population

Four males were observed to father offspring in two separate years but no males were re-sighted within years. Under our basic simulated sampling model, allowing re-sighting within and between years, male population sizes in the range 2300–2700 had the highest probability of producing four re-sightings. If males are only allowed to be re-sighted in separate years, which is more concordant with our observed data, a simulated male population of 1600–2000 gives a re-sighting

Table 2
Estimates of effective population size, N_e (mean, 95% CIs) from the sibship and linkage disequilibrium methods applied to data subsets.

Subset	n	Sibship method	Linkage method
Cousine males	91	157 (117–214)	1781 (725–∞)
Cousine females	172*	236 (189–292)	1080 (720–2087)
Non-Cousine females	102	181 (138–242)	1176 (639–6188)
Cousine all	263*	356 (296–426)	1494 (1013–2746)
All adult females	274*	359 (300–427)	1722 (1143–3363)
All adults	365*	485 (418–562)	2407 (1578–4872)

n = number of genotype individuals.
* Refer to numbers excluding two five-member full-sib groups, bar one, randomly-selected member per group.

Table 3
Posterior distribution summary statistics of parameters estimated by DIY ABC simulations that included a 'vary N_e' scenario. Values for medians give the range covered by three runs of the programme; values for 95% CI are the means of the three 95% CI estimates. Estimates of current N_e obtained from the 'constant N_e' scenario are given for comparison.

Parameter	Median	95% CI
Current N_e ('vary')	4390–4450	406–9747
Historical N_e	901–1110	149–7570
Time since change (generations)	211–220	10–483
Current N_e ('constant')	1020–1150	178–8743

mode matching our observed four re-sightings. We treated the single observed incidence of the same pairing of a specific male and female occurring in two years as an independent re-sighting of the male. Removing this pair from the simulation increases estimates of male numbers to approximately 2800–3700 if intra-year re-sightings are allowed, and 2100–2700 if they are not. Restricting a proportion of males to being biennial did not affect the estimates, but larger population values were indicated if some males were allowed to be more successful in achieving paternity (data not shown).

4. Discussion

Our study of hawksbill turtles in Seychelles indicates that population structure over the area sampled was very low. Slightly higher levels of relatedness amongst Cousine Island females than amongst males may indicate that dispersal is, to some degree, sex-biased. While the various methods used to estimate effective population size provided differing results, the consensus indicated an N_e of approximately 1000–2000, and there was little, if any, evidence of a recent genetic bottleneck. Concordant with this, on Cousine Island, very few males were 'genetically re-sighted' within or between years, indicating that a large male population (>1000) currently exists in the Seychelles area.

The absence of significant genetic population structure between hawksbill nesting beaches across Seychelles suggests that, with respect to nuclear DNA, our samples are drawn from a single, panmictic population. In initial testing, several F_{ST} comparisons that involved Desroches were significant (Table S3), but these did not remain significant after correcting for multiple comparisons, and the programme STRUCTURE did not separate this island into its own genetic group, even when provided with 'island' as a location prior. In terms of breeding females, the genetic population inferred from this data spans at least the 450 km from the inner granitic islands to Alphonse and St. François on the Amirantes Bank. This result is consistent with a previous comparison of females nesting in the Granitics and Amirantes that used a 766 bp section of the mtDNA control region ($\varphi_{ST} = -0.018$, $n_{Granitics} = 47$, $n_{Amirantes} = 25$; Vargas et al., unpublished data), although it should be noted that alternative mtDNA markers, including whole genome sequencing, have identified genetic structure in some marine turtle populations where the variation at the control region did not (e.g. Shamblin et al., 2012; Tikochinski et al., 2012). We further found that juvenile hawksbills feeding around Aldabra, a further 750 km away, were not genetically separable from adults breeding in the Granitics and Amirantes, potentially suggesting a much wider geographic extent to this single population. However, the long migrations of young turtles, which can span entire oceans (e.g. Bowen et al., 1994, 1995), mean that we cannot assume that the juveniles feeding around Aldabra are derived from the small number of adult hawksbills nesting on Aldabra. An alternative possibility is that Aldabra is a feeding ground for young turtles hatched on the granitic and coralline islands. This scenario is supported by molecular evidence for a similar process occurring between Seychelles and Chagos hawksbills (Mortimer and Broderick, 1999; Sheppard et al., 2012), and by the interceptions of a nesting female in the Amirantes and a subadult male in the Granitics that had both been tagged as juveniles at Aldabra (Mortimer et al., 2010). Such long connections between juvenile feeding grounds and source rookeries are also well known from studies of Caribbean hawksbills (Blumenthal et al., 2009; Bowen et al., 2007), a region where molecular data has featured prominently in discussions on cross-border management of hawksbill populations (Godfrey et al., 2007; Mortimer et al., 2007a,b).

The mean genotype assignment index of Cousine Island females was not significantly higher than that of the inferred males, which argues against sex-biased dispersal. However, the distribution of the index differed significantly between the sexes, which may indicate that males and females are of subtly different genetic origins. This is supported by relatedness amongst Cousine Island females being significantly higher than amongst the males. The effect size was small (0.008), but held up to subsampling the female–female dyads to reduce sample size inflation. Is it safe to interpret such a small effect, based on a large sample size, as indicative of meaningful sex-biased dispersal, with males as the more dispersive sex? If sex-biased dispersal were strong, one would expect clearer genetic evidence for a 'non-dispersing' sex. In marine turtles, this is traditionally held to be females returning to breed in their natal areas, a hypothesis that is well-supported by numerous mtDNA studies at coarser geographic scales (reviewed in Bowen and Karl, 2007; Lee, 2008). However, mtDNA and microsatellites do not give contrasting signatures of population structure between the Granitic Seychelles and the Amirantes: we found no population structure between these island groups at microsatellite loci ($F_{ST} = 0.001$), and Vargas et al. (unpub. data; see above) found no mtDNA structure (but see Shamblin et al., 2012; Tikochinski et al., 2012). If the relatedness test is indicative of meaningful sex-biased dispersal, then it must be taking place against a background of high dispersal by both sexes. That female dispersal occurs is supported by hawksbill tagging data from Seychelles: inter-island tag re-sightings are uncommon but not infrequent within the Granitics, and an adult female tagged on Bird Island in the Granitics (03°43′S, 55°12′E) has been observed nesting 280 km away on D'Arros Island (Amirantes group; Mortimer, unpub. data). Such dispersal events need only occur once per generation to reduce population structure to extremely low levels (Wright, 1931). Interestingly, female–female dyads make up a higher proportion of the higher-relatedness dyads than male–male dyads do, and this effect becomes stronger as relatedness increases ($r > 0.45$; Fig. 2). This suggests that the overall relatedness effect may be driven by there being more $r \approx 0.25$ (e.g. half-sib) and $r \approx 0.5$ (e.g. full-sib, parent–offspring) dyads amongst females than amongst males. It therefore seems that there is some degree of natal homing amongst females, greater than amongst males, but that this is not strong enough to generate population genetic structure.

As genetic structure at maternally-inherited mtDNA is normally pronounced across marine turtle populations (Lee, 2008), evidence of low nuclear (i.e. bi-parentally inherited) structure, as seen in our study, is thought to be indicative of sex-biased dispersal. However, explicit molecular tests for sex-biased dispersal are relatively few and do not present a clear single picture, and the use of different molecular markers and sampling regimes makes quantitative comparisons between studies difficult. In perhaps the best example, given that the study used nuclear and mitochondrial DNA and directly sampled both sexes across multiple populations, FitzSimmons et al. (1997b) demonstrated male-biased gene flow amongst green turtle (*Chelonia mydas*) populations along the east coast of Australia. However, mtDNA structure amongst the males suggested that this effect came about through mating on migration, rather than greater dispersal of males per se (FitzSimmons et al., 1997a). Similarly, at the Rocas Atoll green turtle rookery in Brazil, there was no significant mtDNA differentiation between males sampled on offshore courting sites and females sampled while nesting (Naro-Maciel et al., 2012). In contrast, Lee et al. (2007) report fine-scale male-biased dispersal against a background of weak population structure amongst females in the green turtles of Ascension Island. In loggerhead turtles (*Caretta caretta*), Bowen et al. (2005) reported extremely strong mtDNA structure between rookeries in the

south-eastern United States but no significant structure at microsatellites, although they do not use male genotypes in their analyses. In loggerheads in the Cape Verde Islands, Stiebens et al. (2013) also report results indicative of sex-biased dispersal, but their interpretation hinges on genetic differences associated with a single island, and they do not satisfactorily rule out alternative demographic explanations, such as a bottleneck on this island. Finally, in hawksbill turtles on Mona Island, Puerto Rico, Velez-Zuazo et al. (2008) report natal homing in both sexes, based on mtDNA. However, the signature for males was weaker than for females, with 20% of males possessing haplotypes not recorded in the island's females. Overall, perhaps the only generalisation that can currently be made is that sex-biased dispersal varies in extent and magnitude between populations. This assessment is supported by satellite tracking of breeding males, which have been recorded travelling large distances within breeding seasons, passing, and potentially contributing to, a large number of rookeries (Wright et al., 2012b), but have, in other cases, been effectively sedentary (van Dam et al., 2008).

Estimates of N_e ranged from 485 for the sibship method to ca 4400 for the DIY ABC analysis that allowed for a historical change in N_e (see Table 3). While it is debatable as how best to form a quantifiable consensus from these varied estimates, a value in the 1000–2000 range seems a fair summary. This is substantially greater than the figure of 500 often quoted as a minimum for the long-term persistence of current population genetic variation (Franklin, 1980; see also Franklin and Frankham, 1998). The wide nature of confidence intervals for most of the methods used may reflect that accuracy and precision of N_e estimation can decrease as N_e increases (Luikart and Cornuet, 1999; Waples and Do, 2010). Furthermore, both the linkage and sibship methods may be underestimating N_e: the patterns of relatedness observed within the large Cousine Island female dataset may bias both estimates downwards, and, looking at estimates derived from data subsets (Table 2), neither method seems to be levelling off as sample size increases. Although marine turtles violate some of the life history and demographic assumptions of N_e estimation (Hare et al., 2011; Palstra and Ruzzante, 2008; e.g. overlapping generations, which can bias estimates both upwards and downwards), our inferred values are in keeping with census estimates for the region of >2500 females (>1000 nesting females per year, with average female re-migration interval of 2.5 years; Mortimer and Bresson, 1999; Mortimer and Donnelly, 2008), given the genetic evidence that this population is well mixed. If an equal sex ratio is assumed (discussed further below), this gives a relatively high N_e/N ratio of 0.25–0.50 (Frankham, 1995).

The Garza–Williamson index was on the edge of significance ($P = 0.03–0.06$), but the observed values of 0.76–0.84 were far from those that would indicate a recent or strong bottleneck (ca 0.6; Garza and Williamson, 2001). In BOTTLENECK, the two-phase model indicated a heterozygosity deficiency, suggesting a population expansion. Similarly, DIY ABC supported a scenario of N_e expansion, although the timing of this was ambiguous and the programme did not consider this scenario to be more likely than one of constant population size. We thus conclude that there is no clear evidence for a recent or strong population bottleneck. This result is concordant with previous work on leatherback (*Dermochelys coriacea*; Rivalan et al., 2006) and flatback sea turtles (*Natator depressus*; Theissinger et al., 2009), which both found no indication of bottlenecks. Rivalan et al. (2006) argue that their leatherback population is composed of immigrants from a larger metapopulation of unknown bounds, whereas Theissinger et al. (2009) discuss the role of mating systems in maintaining N_e in their flatback population. In the present study on hawksbill turtles, we find evidence that both population connectivity and the mating system play a role in maintaining a high N_e.

Our observations of the hawksbill mating system were consistent with Phillips et al. (2013): season-long sperm storage, predominance of single paternity, and no males fathering the offspring of more than one female within a breeding season. A small number of males were detected in two seasons: two in consecutive seasons and two at two-year intervals (see also Wright et al., 2012a). This includes the single incidence of a particular male–female pairing being observed in two years, which could also arise from inter-year sperm storage by the female. If so, this would be the first time this phenomenon has been documented in wild marine turtles, although it is well known amongst non-marine testudines (see Phillips et al., 2014). We have previously speculated on how the absence of males fertilising multiple females within a year, together with infrequent multiple paternity, may be indicative of a large but dispersed male population (Phillips et al., 2013). The present study, which has extended the sampling across multiple years, supports that conclusion, as even in large, randomly mating populations, one expects occasional re-sightings of males. Our simulations suggest a male population in the 1500–3000 range, which is concordant with the current Seychelles census estimates of >2500 female hawksbills (see above). Interestingly, studies on sex ratios in hatchling and juvenile marine turtles predominantly report female biases (e.g. reviewed in Wright et al., 2012b), whereas our study is part of a growing body of literature arguing that operational sex ratios in adult marine turtles are, if anything, male-biased (Lasala et al., 2013; Stewart and Dutton, 2011; Wright et al., 2012a,b), although it is not clear to what extent this is affected by differences between male and female remigration intervals (Hays et al., 2010; Wright et al., 2012a). There are currently no data available on hatchling or juvenile sex ratios in the Seychelles hawksbill population against which estimates of adult sex ratio (actual and operational) could be compared (Mortimer, pers. comm.). It is also important to note that the historical hunting of hawksbills in Seychelles was markedly female-biased, as females are easier to catch, and yield more 'tortoiseshell' (Mortimer, 1984). The observed adult sex ratio may thus represent a hangover from over-hunting, rather than the stable ratio of a demographically 'healthy' population.

Within and across years, more males than females contributed to the parentage of Cousine offspring (148 v. 128 for all years; 142 v. 128 for Y1–Y3). Reproductive variance amongst these males appears to be low: 121/148 inferred males fertilised all the clutches of a separate, different female within a single season. Indeed, males seem to be sufficient in numbers and/or sufficiently widely distributed/mobile to prevent most individual males from gaining matings/fertilisations with multiple females coming to nest on Cousine (see also Phillips et al., 2013). Were there to be a discrete, repeatable subset of males that dominated paternity, the resulting reproductive skew would significantly reduce N_e. Instead, mating seems likely to be occurring on migration, and/or over a wide geographic area, promoting gene flow and helping keep N_e high.

Hawksbill numbers in Seychelles have declined substantially in the 200+ years since human colonisation (Mortimer, 1984, 2004), part of a global reduction that has seen the species listed as critically endangered (Mortimer and Donnelly, 2008). However, our work indicates that, in Seychelles, this decline has not left a detectable genetic signature: N_e remains high, and high relative to census counts; the population is not inbred (F_{IS} values were not significantly different from zero); and there is no indication of a population bottleneck. It is possible that the population was not reduced sufficiently, or held low for long enough given the long, overlapping generation times of turtles (ca 35 years for hawksbills in the Indo-Pacific; Mortimer and Donnelly, 2008), for severe negative genetic effects to occur. If, as is suggested by our results, Seychelles hawksbill turtles from the Granitic islands to the Amirantes are one panmictic population, this will have played an important role in keeping N_e high and preventing the loss of genetic diversity to bottlenecks in subdivided populations (Frankham, 1995; Wright, 1931). That mating involves a large number of widely dispersed males, thereby promoting gene flow and lowering reproductive variance amongst males, will also help keep N_e high. An alternative explanation for the relatively large N_e and the lack of population bottleneck is that the long generation time of hawksbills has caused a lag in the

reduction in N_e one would expect following overhunting. Thus the high N_e relative to N that we detect may be because the estimated N_e still reflects that of the larger, historical population. That hawksbill numbers are now recovering in Seychelles, especially on protected islands (e.g. Allen et al., 2010), may mean that the population has escaped a serious demographic event with relatively little long-term negative impact on genetic diversity.

Although our results are positive in conservation genetics terms, we should not be complacent with respect to ongoing hawksbill conservation in the region: although population sizes are increasing in protected areas (e.g. Allen et al., 2010), the species remains substantially reduced in distribution and numbers compared with recent history (Mortimer, 1984, 2004). Extension of beach protection may be a productive strategy, given the evidence that females may disperse and lay clutches on different nesting beaches, thereby facilitating the colonisation of currently unoccupied sites. The lack of genetic differentiation between juveniles in Aldabra and breeding adults in the Granitic and coralline islands highlights how marine turtle conservation needs to involve the protection of different, potentially very distant, areas for different life stages of a single population (e.g. Blumenthal et al., 2009; Bowen et al., 1994, 1995, 2007; Hawkes et al., 2006; Mortimer et al., 2010). The future for Seychelles hawksbills is harder to predict. For example, in the face of global climate change, the temperature-dependent sex determination of turtles is an obvious source of vulnerability that may require an adaptive response (Hawkes et al., 2009; Wright et al., 2012b). However, a large N_e, gene flow spanning a wide geographic area, and no indication of a recent bottleneck all suggest that hawksbills in the Seychelles are in a relatively healthy position, genetically speaking, to adapt to the considerable challenges they face.

Finally, this study reinforces the utility of molecular techniques for providing valuable insight into the life histories and demography of difficult-to-study species, and underlines the importance of understanding genetic processes in species of conservation concern. Our results will be of particular interest to biologists and managers focusing on long-lived, slow-to-mature, migratory species, especially marine species and species with wide ranges and low population densities.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jembe.2014.02.012.

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