Vol. 716: 93–105, 2023
https://doi.org/10.3354/meps14367

MARINE ECOLOGY PROGRESS SERIES
Mar Ecol Prog Ser

Published August 10

Twenty-year changes in the composition of a mixed stock of foraging green turtles in the Yaeyama Islands of Japan

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ABSTRACT: Recent studies have shown an increasing mean straight carapace length (SCL) of foraging green turtles *Chelonia mydas* around the Yaeyama Islands, Japan, compared to that of 20 yr ago. This trend is attributed to reduced juvenile mortality resulting from a decline in sea turtle fisheries. In this Yaeyama foraging aggregation, the proportions of turtles originating from different populations (genetic stocks) are known to vary according to size; for turtles originating from the Ryukyu Archipelago and Ogasawara (Japanese populations), the proportion in the 50–70 cm SCL size class is higher than that in the <50 cm SCL size class. Here, we compared the natal origin compositions of the Yaeyama foraging aggregation from 2 sampling periods (1997–1999 and 2016–2018) across 2 size categories (<55 and >55 cm SCL) and investigated the relationship between the size increase and changes in natal origins. The results showed that the proportion of the Japanese populations has recently increased compared to that 20 yr ago. The size distribution, based on the natal origin of each turtle in 2016–2018 estimated using single nucleotide polymorphisms, showed that the proportion of populations at latitudes lower than Japan with a ≥55 cm SCL decreased substantially, whereas the proportion of the Japanese populations remained stable between size categories. These results suggest a link between the size increase and the increase in Japanese turtles. This study also provides the detailed composition of the Yaeyama foraging aggregation, including the size distributions and sex ratios of contributing populations.

KEY WORDS: *Chelonia mydas* · Foraging aggregation · Twenty-year change · Size increase · Stock composition

1. INTRODUCTION

Anthropogenic effects on marine turtles have threatened many populations for centuries. However, conservation efforts that began in the late 1900s have successfully restored the annual number of nests in many populations (Mazaris et al. 2017). For green turtles *Chelonia mydas*, population recovery has recently affected the seagrass ecosystem, which comprises their main foraging ground (FG), in some regions due to grazing pressure. For example, overgrazing by green turtles has led to the loss of seagrass meadows in Bermuda and the northern Indian Ocean (Fourqurean et al. 2010, Gangal et al. 2021) and has accelerated the replacement of native seagrasses by invasive species in the Caribbean (Christianen et al. 2019).

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Green turtle foraging aggregations often consist of individuals from multiple distant populations (Joseph et al. 2016, Piovano et al. 2019), while some aggregations are composed of one or a few populations (Dutton et al. 2008, Naro-Maciel et al. 2014). As turtles mature, they exhibit foraging site fidelity to their main FG (Musick & Limpus 1997), although some FGs serve as temporary foraging sites for juveniles as developmental habitats (Meylan et al. 2011). As a result, size distributions vary among FGs, with some dominated by sub-adults and adults (Joseph et al. 2016), while others are predominantly occupied by juveniles (Pilcher 2010, Sterling et al. 2013). In green turtles, which have a slow growth rate, temporal changes in nesting populations are expected to manifest as differences in the growth stages of individuals within FGs. The recovery of nesting turtles at specific rookeries can alter the demographics of the foraging aggregations. Therefore, understanding the demographic changes within FGs, including shifts in mixed stock compositions, will be crucial for achieving balanced conservation efforts and effective ecosystem management.

Like other marine turtles, nesting green turtles are genetically differentiated between remote sites owing to natal philopatry (Jensen et al. 2013). Mitochondrial DNA (mtDNA) haplotype data have been collected from rookeries worldwide to assess the connectivity among rookeries and define populations (Jensen et al. 2019). Haplotype frequencies in populations have been widely utilized to estimate the connectivity between nesting sites and FGs using mixed stock analysis (MSA) (Pella & Masuda 2001, Bolker et al. 2007). MSA enables the determination of population compositions within foraging aggregations based on the distinct frequencies of mtDNA haplotypes among populations.

The southern islands of Japan (the Ryukyu Archipelago [Ryukyus]) and the Ogasawara Islands comprise the northernmost green turtle nesting sites in the northwestern Pacific (Kikukawa et al. 1999, Kondo et al. 2017, Okuyama et al. 2020). There are several genetically distinct populations of green turtles in Japan (Nishizawa et al. 2011, 2013, Hamabata et al. 2014). In addition, the coastal area of the Japanese Archipelago provides foraging habitats (Shimada 2009, Nishizawa et al. 2013, Fukuoka et al. 2015, Hamabata et al. 2015). Among the FGs in the Japanese Archipelago, the FG around the Yaeyama Islands in the southern Ryukyus (Fig. 1) is relatively well studied. Turtles in this FG comprise mixed-size aggregates, ranging from post-pelagic juveniles with a straight carapace length (SCL) of approximately 35–40 cm to adult-sized individuals (Kameda et al. 2017). Additionally, the mean size of foraging turtles has increased by 2–4 cm over the past 20 yr (Kameda

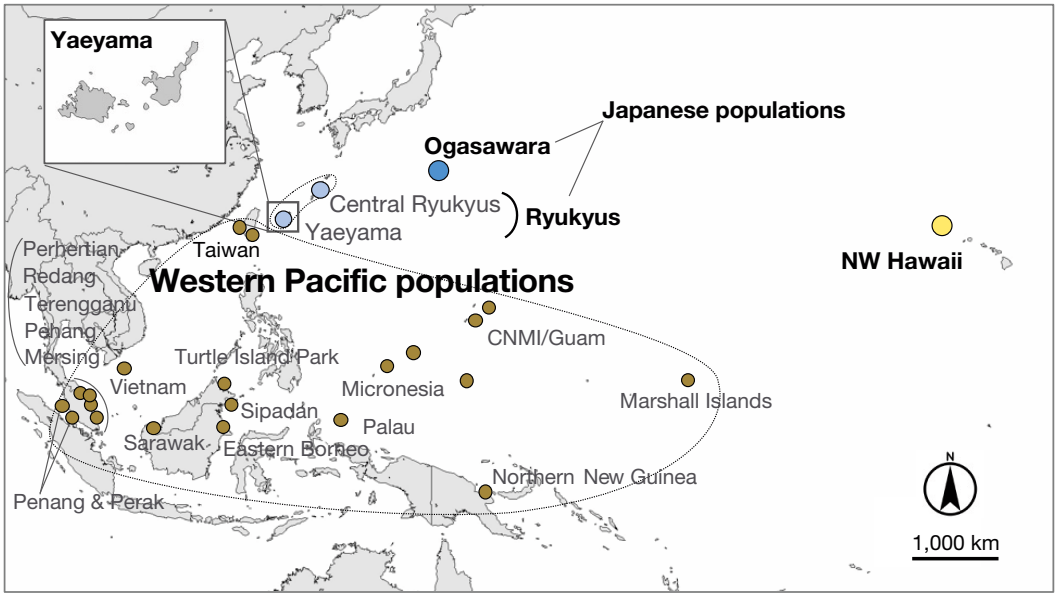

Fig. 1. Study area. Colored dots show the green turtle rookery sites in the northwestern Pacific and northwestern (NW) Hawaii. The inset provides an enlarged map of the study site in the Yaeyama Islands. The nesting sites of the Western Pacific populations outside of Japan in the regional mixed-stock analysis and group assignments are indicated by brown dots and grouped by a dotted line. The populations from the Ryukyus formed 1 group and are shown by light blue dots and circled with a dotted line. The Ogasawara and NW Hawaii populations are highlighted in blue and yellow, respectively

et al. 2017, 2019), and the annual abundance of foraging individuals has been recently increasing in this FG (Kameda et al. 2023). The increase in size is believed to be a result of the decline in juvenile marine turtle fisheries around the Yaeyama Islands, which were previously operated by licensed fishermen for food (Kameda et al. 2017). MSA studies have shown that turtles in this FG originate from rookeries in Japan, as well as the North Pacific (Nishizawa et al. 2013), with the contributing populations varying by size class. Specifically, the proportion of Japanese turtles in the 50–70 cm SCL class has increased (Hamabata et al. 2018). The observed increase in the mean size of foraging turtles could be linked to the increase in individuals from Japanese rookeries. Here, we compared the mixed stock composition of turtles in the Yaeyama FG with that observed 20 yr ago and tested the hypothesis that the recent increase in mean size might be attributed to an increase in turtles from Japanese rookeries.

This study employed conventional MSA using mtDNA and group assignments based on high-throughput multiplexed inter-simple sequence repeat (ISSR) genotyping by sequencing (MIG-seq), which utilizes genome-wide single-nucleotide polymorphisms (SNPs) (Suyama & Matsuki 2015). Since only mtDNA data are available for samples collected more than 20 yr ago (in 1997–1999) (Hamabata et al. 2009), we conducted a conventional MSA on samples collected in 1997–1999 and in 2016–2018 to compare the overall demographic composition. In this analysis, it was not possible to determine the natal origin of each individual owing to the presence of several common haplotypes shared among multiple related populations within Japan and other Western Pacific regions. Subsequently, we utilized genome-wide SNPs to estimate the natal origin of each individual collected in 2016–2018 and compared the size distributions among populations within the Yaeyama foraging aggregation. The samples collected from 2016–2018 were obtained from turtles whose sex had been determined using laparoscopy in a previous study (Kameda et al. 2019). Interestingly, it has been observed that the proportion of males is higher in the ≥55 cm SCL size class than in the <55 cm SCL size class within this FG (Kameda et al. 2019). This disparity might indicate variations in sex ratios among the contributing populations. With the available sex information determined using laparoscopy for each individual, we further examined the sex ratio of each contributing population within the Yaeyama foraging aggregation, providing a report on the detailed demographic composition of foraging turtles in this region.

2. MATERIALS AND METHODS

2.1. Samples

We analyzed tissue samples collected from 104 foraging turtles around the Yaeyama Islands (Fig. 1) from 2016 to 2018 in a previous study by Kameda et al. (2019). These tissues were collected when tags were attached to individuals in a mark–recapture research program. In addition, genetic data (mtDNA) from 123 turtles collected during 1997–1999 were obtained from the appendix of Hamabata et al. (2009) (Table S1 in the Supplement at www.int-res.com/articles/suppl/m716p093_supp.pdf). Tachikawa (1991) reported that the minimum sizes (SCL) for a turtle to be an adult in Ogasawara were 79.4 cm for males and 82.1 cm for females. We used these sizes to classify turtles collected in 2016–2018 as either immature or mature. The number of individuals used in this study in each period is provided in Table 1.

Genomic DNA was extracted from the 2016–2018 samples using DNeasy Blood and Tissue Kits (Qiagen), and the haplotype of the mtDNA control region was determined using an ABI 3730xl DNA analyzer with the primers LCM15382 and H950 (Abreu-Grobois et al. 2006). The Pacific standard names of all haplotypes were identified using an NCBI BLAST search. Of the 104 turtles, the haplotypes of 12 turtles clearly indicated that they originated from outside Japan (i.e. Micronesia/Marshall Islands, Southeast Asia, and northwestern Hawaii). These turtles were used as origin-known samples in the group assessment. The group assessment also included preserved DNA samples from 37 turtles captured from nesting sites or identified based on their regionally endemic mtDNA haplotypes. These turtles originated from the Ogasawara Islands (n = 10), the Ryukyus (n = 22), and Micronesia/Marshall Islands (n = 5) (Fig. S1).

2.2. MSA

The mtDNA haplotype data of the 123 samples from 1997–1999 published by Hamabata et al. (2009)

Table 1. Sample details of green turtles used in this study. The numbers in brackets represent the samples used in the mixed-stock analysis. nd: not determined

	Female	Male	Total
1997–1999	nd	nd	123
2016–2018	67 (65)	37 (36)	104 (101)

and 101 samples from 2016–2018 in this study were analyzed to estimate the contribution of the nesting populations to the present foraging aggregation with 95% credible intervals. The analysis used the conventional Bayesian method (Pella & Masuda 2001). The haplotype data of the nesting sites of the 21 distinct populations, which are considered to be related to the FG around the study site, were obtained from previous studies (Nishizawa et al. 2011, 2013, Dutton et al. 2014, Hamabata et al. 2014, 2020, Jensen et al. 2016a) and compiled as baseline data. The lengths of the haplotypes were truncated in ~380 bp sequences owing to the limited data available in some populations. The contributions were independently estimated using all 21 regional populations. Previous studies have suggested the presence of multiple distinct genetic stocks within the Yaeyama region (Nishizawa et al. 2011) and Central Ryukyus. However, the exact boundary within the Central Ryukyus remained unclear (Hamabata et al. 2014). Therefore, the Yaeyama and Central Ryukyus were treated as separate groups and subjected to MSA to determine their respective regional contributions. To summarize the broad regional contributions and compare the results with the subsequent group assignments based on genome-wide SNPs, the regional MSA was also performed based on the following 4 regions: the northwestern Pacific region other than Japan (the Western Pacific populations), Ryukyus, Ogasawara, and NW Hawaii. We performed Markov chain Monte Carlo simulations for 60 000 iterations with a burn-in of 30 000 in 4 chains. The convergence of all chains was confirmed based on a Gelman and Rubin shrink factor <1.2. The estimation was performed on the basis of the total number of samples in each period. All MSA estimations involved an uninformative prior because previous studies revealed that informative priors (weighted by population size) yielded similar results in this FG (Nishizawa et al. 2013, Hamabata et al. 2018).

2.3. Procedure for MIG-seq

The concentration of genomic DNA was measured using a NanoDrop spectrophotometer (Thermo Scientific) and was adjusted to 1 ng μl^{-1}. The genomic libraries were prepared according to the protocol described by Suyama et al. (2022). Genome-wide non-repetitive regions between the various simple sequence repeats were amplified by the first PCR using multiplex inter-simple sequence repeat primers. A second PCR was performed to add the complementary sequences of the binding sites in an Illumina sequencing flow cell and the indices (barcodes) for each sample to the first PCR products. The libraries were purified and sequenced on an Illumina MiSeq sequencer (MiSeq Control Software v2.0.12; Illumina) with a MiSeq Reagent kit v3 (150 cycles; Illumina) based on 80 bp paired-end reads. All read data were deposited into the NCBI Sequence Read Archive (BioProject PRJDB13199; BioSample Accession SAMD00446999–SAMD00447142). A turtle with haplotype CmP2.1 was excluded from the analyses because it is highly probable that the individual originated from NW Hawaii, even without the group assignment.

2.4. SNP detection

Low-quality reads were filtered from the samples using Trimmomatic v.0.35 (Bolger et al. 2014) with settings of the sliding window of 4 bases with an average quality of 15. We also trimmed 6 and 3 bases from the beginning and end of the reads, respectively. Any reads with fewer than 71 bases were discarded. SNP detection was performed using STACKS version 2.3 (Rochette et al. 2019) and a reference-based pipeline. A reference genome (NCBI BioProject: PRJNA561941, *Chelonia mydas* genome assembly rCheMyd1.pri.v2) (Bentley et al. 2023) was used. The procedure used for the reference-aligned analyses was as follows: quality-filtered reads were mapped to the reference genome using the BWA-MEM algorithm Burrows-Wheeler Alignment Tool (Li & Durbin 2009) version 0.7.13. The results of mapped reads were outputted in 'bam' format using 'samtools' version 1.9 (Li et al. 2009). The reads that were mapped to and properly paired with the reference genome were included. Any unmapped reads and secondary alignments were excluded. Alignments with a mapping quality smaller than 20 were skipped. These procedures were executed with the command 'samtools view -b -q 20 -f 0x0002 -F 0x0004 -F 0x0008 -F 0x0100'. After sorting the alignments by left-most coordinates ('samtools sort' command), we ran 'gstacks —rm-pcr-duplicates' of STACKS to build loci from the aligned paired-end data to remove any PCR duplications. The 'populations' modules of STACKS were first run with 48 samples from which the natal origins were known in advance. As reported in a previous study, turtles from northwestern Pacific populations outside of Japan (i.e. Southeast Asia and Micronesia/Marshall Islands) grouped together into 1 cluster owing to their genetic similarity or small number of samples

(Hamabata et al. 2020). In this analysis, we treated the samples from these populations as 1 wider regional group, corresponding to the Western Pacific populations in the regional MSA. Furthermore, when analyzing the SNPs, it has been demonstrated that the population structure of the Ryukyus (Yaeyama and Central Ryukyus) shows overall continuity, making it challenging to distinguish Yaeyama and the Central Ryukyus from each other (Hamabata et al. 2020). Hence, the natal origins of individuals from the Ryukyus were estimated using SNPs without subdividing the regions. Turtles (n = 48, excluding 1 sample from NW Hawaii) with a known natal origin were classified based on 3 regions, namely the Western Pacific (n = 16), Ryukyus (n = 22), and Ogasawara (n = 10) populations. In this 'populations' module, the minimum allele frequency threshold was set to 0.05, the proportion of shared SNPs among individuals in a population (–r option) was set to 0.8 (80%), and the minimum number of populations with a locus (–p option) was set to 2. We tried the –r option using increments of 0.1 from 0.6 to 0.9. Because 0.6 and 0.7 resulted in more missing SNPs and missing sites were replaced by the mean allele in the discriminant analysis of principal components (DAPC), the population structure was obscure. Moreover, with the highest threshold of 0.9, the number of SNPs decreased to fewer than 1000, obscuring the structure. We thus used a –r of 0.8 and extracted 1676 SNPs that showed the highest proportion of overall correct assignments (0.938) to 3 populations for 48 samples of a known origin. The commands that extract SNPs are available in the GitHub repository (https://github.com/tmkhmbt/foraging_turtle_analysis_with_SNPs). The population structures of the 3 regions were visualized in a scatterplot and a histogram. The number of principal components (PCs) to be retained was explored based on cross-validation, which was implemented in the DAPC (Jombart et al. 2010) 'adgenet' 2.1.8 package for R version 4.2.2, using a training set size of 85% and 30 replicates. We used 'adgenet' according to the tutorial (Jombart & Collins 2015).

2.5. Natal origin estimation

For all samples, the 1676 SNPs that were at the same loci as those used to examine the population structure of samples of a known natal origin were extracted using the whitelist (–W) option of the 'populations' module. The group assignments of samples of an unknown origin were independently performed on the samples with common Japanese haplotypes (n = 49) and on samples with widespread and orphan haplotypes (n = 43) using the 'find.clusters' function in 'adgenet'. Former group assignments were carried out using 81 individuals, including samples of a known origin from the Ryukyus and Ogasawara populations, and the number of PCs for which the Bayesian information criterion (BIC) became lowest at 2 clusters was explored by running *k*-means. The latter group assignments were carried out using 91 individuals, including samples of a known origin from the Western Pacific, Ryukyus, and Ogasawara populations, and the number of PCs for which the BIC became lowest at 3 clusters was explored by running *k*-means. The number of PCs with the smallest BIC at 2 clusters ranged from 18 to 24 in the group assignment for turtles with common Japanese haplotypes. Whereas all Ogasawara samples were assigned to a single cluster, some samples from the Ryukyus were assigned to the same cluster with the Ogasawara samples. The number of PCs with the smallest BIC at 3 clusters ranged from 17 to 20 in the group assignment for turtles with widespread and orphan haplotypes. All Ogasawara samples were also assigned to a single cluster in this assignment. However, some samples from the Ryukyus were assigned to the same cluster with the Ogasawara populations or with the Western Pacific populations. Some samples from the Western Pacific populations were assigned to the cluster dominated by those from the Ryukyus. Samples assigned to populations different from their known origin based on the group assignment were defined as 'mismatched samples.' Since the number of mismatched samples slightly changed among the number of PCs, the number of PCs retained was determined to minimize the number of mismatched samples. Finally, we determined the numbers of retained PCs as 23 PCs in the group assignment for the turtles with common Japanese haplotypes and 19 PCs in the group assignment for the turtles with widespread and orphan haplotypes. The population structure of the 140 samples based on the results of group assignments was also visualized in a scatterplot and a histogram. The number of principal components (PCs) to be retained was explored based on the cross-validation under the same condition as the first 48 origin-known samples. If samples from the foraging aggregation, which were treated as samples of a known origin, resulted in mismatched group assignments, they were classified based on their known origins (estimated using mtDNA or the actual sampling site) and included in the subsequent statistical analyses.

2.6. Statistical analysis

The Shapiro–Wilk test was used to test the normality of sizes before the statistical analysis of size differences among natal origins and between sexes, and the present data deviated from normality ($W = 0.910$, $p << 0.001$). Kruskal–Wallis tests were then used to test size differences among natal origins, and Welch's *t*-test was used to test size differences between sexes. The natal origins of turtles in <55 and ≥55 cm SCL size classes were tested by performing a chi-squared test. The 55 cm SCL cut-off was determined based on the mean size of current foraging turtles in this FG. The mean size of the total samples in this study was almost the same (54.8 cm, SD = 11.8 cm). This size classification is considered reasonable based on the sharp decline in the histogram around this size. In addition, sex ratios (% female) based on total samples and samples only from the ≥55 cm SCL size class were statistically compared using chi-squared tests. All statistical analyses were conducted using R.

3. RESULTS

3.1. Stage and mtDNA haplotype of 2016–2018 samples

One hundred turtles (96.2%) were immature-sized, and 4 turtles (3.8%) were mature-sized. All mature-sized turtles were captured outside of the reproductive (mating and nesting) season; thus, they were not breeding migratory turtles. We identified 17 haplotypes in 104 samples of green turtles that were foraging around the Yaeyama Islands. All haplotypes had been previously reported and referenced in GenBank (Table 2). Three turtles had orphan haplotypes (CmP79.1 and CmP213.1) and were excluded from the MSA because they have not been found in any rookeries, and candidate natal origins could not be estimated. These turtles with orphan haplotypes were analyzed together with turtles with widespread haplotypes (CmP20.1, CmP49.1, and CmP77.1) in group assignments using SNPs. The common Japanese haplotypes classified for group assignments were CmP39.1, CmP50.1, CmP53.1, CmP54.1, CmP121.1, and CmP127.1.

3.2. MSA by mtDNA

The results of the MSA based on mtDNA haplotype data of all 21 populations showed that Micronesia had the highest contribution to samples from 1997–1999 and 2016–2018; however, there was a considerable decline in contribution in more recent samples, from 0.39 to 0.23 (Table S2, Fig. S2). Furthermore, compared to those of samples in 1997–1999, the contributions of Yaeyama and Northern New Guinea increased by 0.05 or more in 2016–2018, whereas those of the Marshall Islands decreased by 0.05 (Table S2, Fig. S2). However, the credible intervals included 0, except for the results of Ogasawara, Central Ryukyus, Micronesia, and NW Hawaii, based on the 1997–1999 samples, and Ogasawara and Micronesia based on the 2016–2018 samples. The regional MSA showed that the top 3 population contributors (in descending order) were the Western Pacific, Ogasawara, and the Ryukyus in 1997–1999 (Fig. 2a; Table S3). In 2016–2018, the top 3 population contributors (in descending order) were the Western Pacific, Ryukyus, and Ogasawara (Fig. 2b; Table S3).

3.3. Origin estimation based on SNPs

The genetic structure of 48 samples of a known origin from the 3 regions using 1676 SNPs is visualized in Fig. 3. Ten PCs were retained based on cross-validation.

The group assignments of the 49 turtles with common Japanese haplotypes showed that 24 turtles

Table 2. Mitochondrial DNA haplotype frequencies of the green turtles captured during 2016–2018 in the foraging ground around the Yaeyama Islands, Japan. Asterisks represent the haplotypes classified as common Japanese haplotypes (*) and widespread haplotypes (**) in this study

Haplotype	GenBank no.	Female	Male
CmP2.1	KC306650.1		1
CmP20.1**	AB819806.1	22	9
CmP32.1	KF311749.1	3	2
CmP39.1*	AB819807.1	12	9
CmP49.1**	AB819808.1	3	5
CmP49.3	KJ502572.1	1	
CmP50.1*	AB819809.1	11	5
CmP53.1*	AB819810.1	2	
CmP54.1*	AB819811.1	3	2
CmP61.1	KF311755.1	2	1
CmP77.1**	KF311759.1	1	
CmP79.1	AB896712.1	1	1
CmP87.1	KJ502589.1	1	1
CmP121.1*	AB819813.1	2	
CmP127.1*	AB856321.1	1	1
CmP130.1	AB973567.1	1	
CmP213.1	AB973569.1	1	
Total		67	37

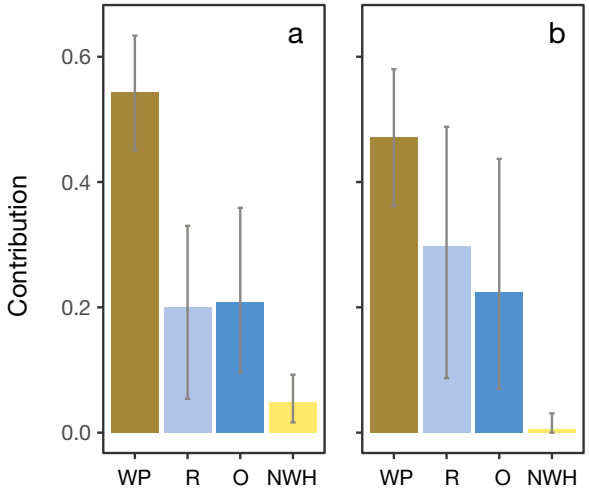

Fig. 2. Results of the mixed-stock analysis (MSA) using mitochondrial DNA from the samples collected in (a) 1997–1999 and (b) 2016–2018. WP: Western Pacific; R: Ryukyus; O: Ogasawara; NWH: northwestern Hawaii populations. Error bars represent 95% credible intervals. Values for this figure are provided in Table S3

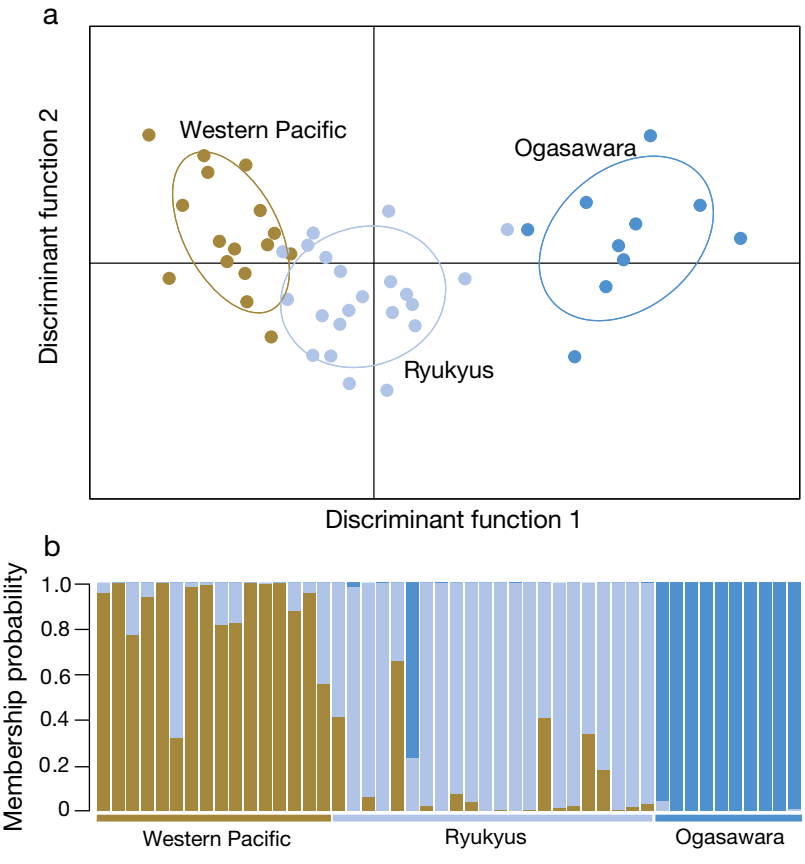

Fig. 3. (a) Structural patterns of natal populations and (b) admixed pattern of each individual. Both structures were generated from discriminant analysis of principal components (DAPCs) based on the 3 regional nesting groups and 1676 single-nucleotide polymorphisms (SNPs)

were assigned to the cluster dominated by Ogasawara, and 25 turtles were assigned to the clusters dominated by the Ryukyus (Fig. S3). In this analysis, 3 turtles for which the origin was known to be the Ryukyus were assigned to the clusters dominated by Ogasawara, indicating a 13.6% mismatch possibility. The group assignments of the 43 turtles with widespread and orphan haplotypes showed that 3 turtles were assigned to the cluster dominated by Ogasawara, 8 turtles were assigned to the clusters dominated by the Ryukyus, and 32 turtles were assigned to the clusters dominated by Western Pacific populations (Fig. S4). In this analysis, 4 turtles with a known origin from the Ryukyus were assigned to clusters dominated by Ogasawara, 2 turtles with a known origin from the Ryukyus were also assigned to the clusters dominated by the Western Pacific populations, and 4 turtles with a known origin from the Western Pacific populations were assigned to the clusters dominated by the Ryukyus. The mismatch possibility of the Ryukyus to Ogasawara was higher (18.2%) than that in the analysis of the common Japanese haplotypes. The mismatch possibility of the Ryukyus to the Western Pacific populations was approximately 9.1%, and that of the opposite relationship was approximately 25.0%. The cluster analysis revealed the following: 12 males and 31 females were estimated to be turtles from Western Pacific populations; 15 males and 18 females belonged to the Ryukyus population; and 9 males and 18 females were from the Ogasawara population. Including 1 individual from NW Hawaii that was not included in the group assignment based on SNPs, the estimated contributions of the Western Pacific, Ryukyus, Ogasawara, and NW Hawaii regions were 0.41, 0.32, 0.26, and 0.01, respectively, among total samples. In addition, they were 0.5, 0.27, 0.23, and 0.00 in the <55 cm SCL samples and 0.29, 0.38, 0.31, and 0.02 in the ≥55 cm SCL samples (Fig. 4; Table S4). Differences in results between mean values of the MSA in 2016–2018 samples and group assignments were 0.07 for the Western Pacific, 0.03 for the Ryukyus, and 0.04 for Ogasawara. The population structures of all 140 samples based on the

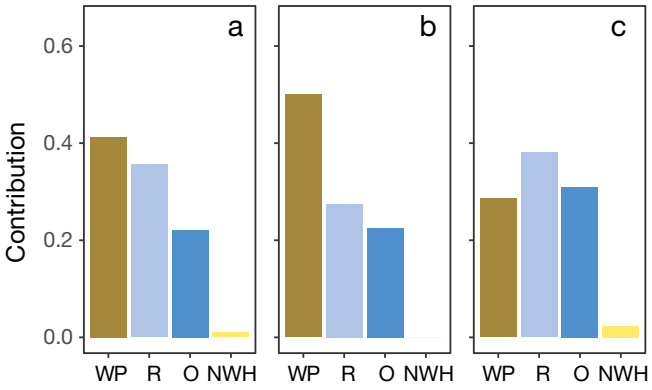

Fig. 4. Results of regional contributions calculated from 103 individuals whose natal origins were estimated based on 1676 single-nucleotide polymorphisms (SNPs) and 1 individual whose mitochondrial DNA was estimated to be from NW Hawaii for (a) total samples collected in 2016–2018, (b) samples in the <55 cm straight carapace length (SCL) size class, and (c) samples in the ≥55 cm SCL size class. WP: Western Pacific; R: Ryukyus; O: Ogasawara; NWH: northwestern Hawaii. Values for this figure are provided in Table S4

results of group assignments are visualized in Fig. S5. Thirty PCs were retained based on cross-validation.

3.4. Size and sex composition

The sizes of foraging turtles in 2016–2018 ranged from 37.5 to 103.5 cm SCL. The peak of the histogram was 45–50 cm SCL (Fig. 5a–c). The size of the smallest turtle in the present sample set was 37.5 cm SCL, and it was a male estimated to be from Ogasawara (Fig. 5d). The mean sizes of the female and male turtles from Western Pacific populations were 53.2 ± 13.4 and 52.2 ± 8.04 cm SCL, those from the Ryukyus were 53.9 ± 11.3 and 59.7 ± 12.4 cm SCL, and those from Ogasawara were 53.9 ± 8.97 cm SCL and 59.4 ± 14.3 cm SCL, respectively (Fig. 5d). Turtles with <55 cm SCL included 31 individuals in Western Pacific populations (9 males, 22 females), 17 individuals in the Ryukyus (6 males, 11 females), and 14 individuals in Ogasawara (3 males, 11 females). Turtles with ≥55 cm SCL included 12 individuals in Western Pacific populations (3 males, 9 females), 16 individuals in the Ryukyus (9 males, 7 females), and 13 individuals in Ogasawara (6 males, 7 females) (Fig. 5e). There were no significant differences in size among turtles with different origins (Kruskal–Wallis tests, p = 0.174) or between sexes (Welch's *t*-test, p = 0.146). In comparisons of the numbers of individuals between size classes, i.e. between <55 and ≥55 cm SCL, there were no significant differences among the 3 populations (χ^2 = 4.36, df = 2, p = 0.113). However, the compositions of size classes differed significantly between the Western Pacific and Japanese populations, which are a compilation of the Ryukyus and Ogasawara populations, with the Japanese populations having a greater proportion of individuals of the ≥55 cm SCL size class (χ^2 = 4.36, df = 1, p = 0.037). Sex ratios (% female) of total samples of each natal origin were 72.1% in the Western Pacific populations, 54.5% in the Ryukyus, and 66.7% in Ogasawara. Sex ratios of samples only of the <55 cm size class were 71% in the Western Pacific populations, 65% in the Ryukyus, and 79% in Ogasawara, and those of samples only of the ≥55 cm SCL size class were 75% in Western Pacific populations, 44% in the Ryukyus, and 54% in Ogasawara. There were no significant differences in sex ratios (χ^2 = 2.57, df = 2, and p = 0.277 for total samples; χ^2 = 0.716, df = 2, and p = 0.699 for samples of the <55 cm SCL size class; and χ^2 = 1.47, df = 2, and p = 0.481 for samples of the ≥55 cm SCL size class). We also tested differences between the Western Pacific populations and combined Japanese samples (Ryukyus and Ogasawara) in sex ratios but found no significant differences.

4. DISCUSSION

4.1. Variation in the foraging aggregation over 2 decades

The present study provides the first report of a 20 yr change in the contributions of natal populations to a green turtle foraging aggregation in the Northwest Pacific. In the regional MSA, compared to that in the 1997–1999 samples, the contribution of turtles from the Ryukyus had increased in 2016–2018, while the contribution of Western Pacific populations had decreased. The MSA results based on 21 populations indicated that the increase in the contribution of the Ryukyus was largely attributable to the local Yaeyama populations. Among the Western Pacific populations, there was an increase in the contributions from northern New Guinea; however, the overall contribution decreased owing to a large decrease in the contribution from Micronesia. In the southern Caribbean FG, the population composition in the foraging aggregation experienced decadal changes due to differences in population recovery between northwestern and eastern Caribbean rookeries (van der Zee et al. 2019). The number of nesting females is indeed increasing in Yaeyama (Okuyama et al. 2020). However, it is also increasing in Ogasawara (Kondo et al. 2017) and the Central Ryukyus (Ministry of the

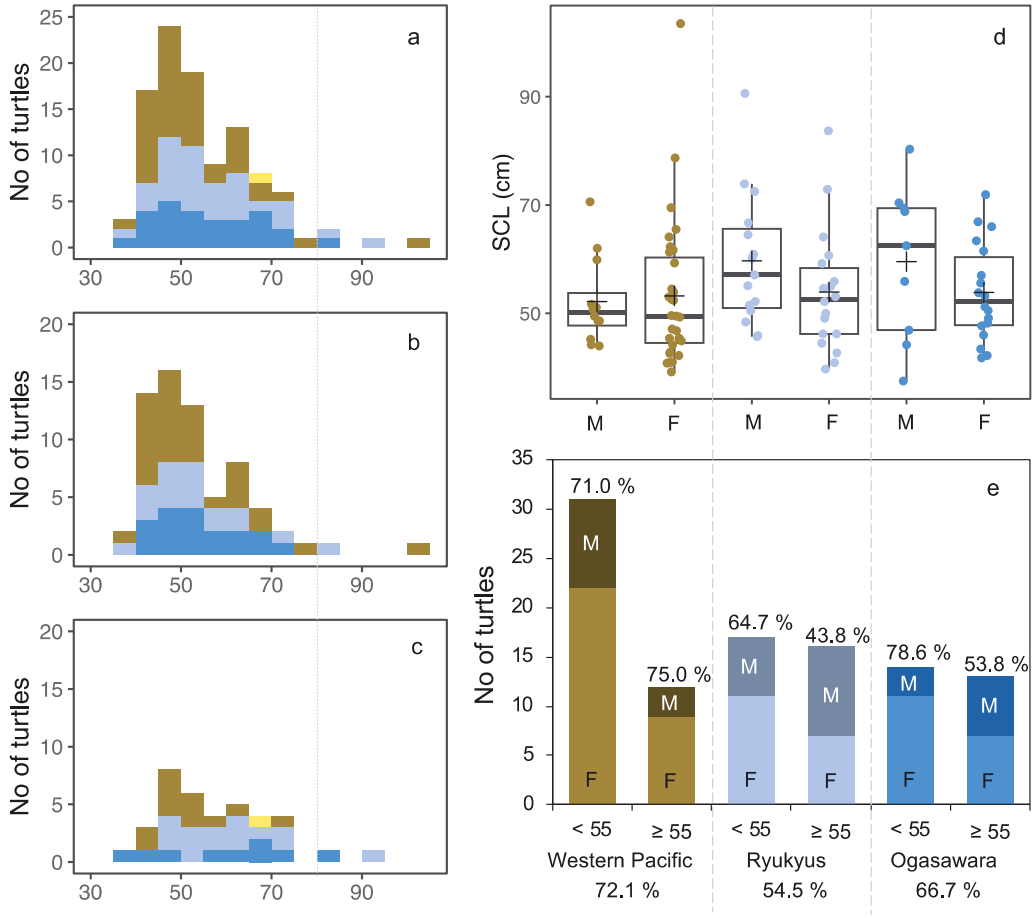

Fig. 5. Sizes of foraging turtles collected in 2016–2018. (a) Total individuals, (b) females, and (c) males are colored according to the estimated natal origins (see Fig. 1). The dashed gray line in panels a–c indicates the boundary between mature-sized and immature-sized samples. (d) Size variation in turtles of different natal origins based on sex (F: female; M: male). SCL: straight carapace length. (e) Numbers of females and males of the 2 size classes, i.e. <55 and ≥55 cm SCL. Males (M) are stacked on females (F) with darker colors. Values above bars indicate the proportion of females in each size class, and those below the *x*-axis labels indicate the proportion of females among all individuals estimated to be from that region

Environment Japan 2014). There are currently no reports available on the population trends in Micronesia and Northern New Guinea. Therefore, additional data are required to assess the impact of population recovery at rookeries on the changes in the population composition within the Yaeyama FG. Importantly, the findings of this study strongly support the possibility that the 20 yr change in the population composition of the Yaeyama FG is associated with an increase in body size.

4.2. Relationship between the recent increase in body size and natal origins

The results of group assignments based on genome-wide SNPs showed slightly higher contributions from the Ryukyus and Ogasawara populations and slightly lower contributions from the Western Pacific populations compared to the results of the regional MSA. The disparity between the MSA and group assignments could be attributed to the misassignment of some samples or the exclusion of 3 samples with orphan haplotypes in the MSA, for which the origins were estimated as the Ryukyus (n = 1) and Ogasawara (n = 2) in the group assignment. There is some uncertainty in the results of the group assignment. However, certain trends in each population will be discussed to explore the relationship between body size and natal origins in the Yaeyama FG.

The average sizes of the turtles from the Ryukyus and Ogasawara were not significantly larger than those of the Western Pacific individuals. However, there was a significant difference in the number of turtles classified into 2 size classes among the regions. The proportion of turtles from the Western Pacific

populations was greater in the <55 cm SCL size class, while the proportion of turtles from combined Japanese populations increased in the ≥55 cm SCL size class. This finding is consistent with a previous MSA study that reported an increased contribution of Japanese turtles in the 50–70 cm SCL size class (Hamabata et al. 2018). The previous study speculated that the increased contribution of Japanese turtles in the 50–70 cm SCL size class might be due to increased recruitment from FGs along the coasts of western Japanese Main Islands, where turtles from Japan are dominant. However, there is currently insufficient mark–recapture data from the western Japanese Main Islands to support this hypothesis (Sea Turtle Association of Japan unpubl. data). The tendency of only the Western Pacific populations to be biased towards the smaller size class suggests differences in habitat use within the Yaeyama FG between the Western Pacific and Japanese populations.

Some green turtle FGs are dominated by immature individuals, and individuals in such FGs are believed to undergo developmental migration (Meylan et al. 2011). However, in certain FGs, juveniles exhibit site fidelity within a specific area over many years (Musick & Limpus 1997). The capture–mark–recapture study in the Yaeyama FG revealed that more than 90% of recaptured turtles remained within the area, indicating long-term residency for many individuals (Kameda et al. 2017). These results, along with the findings of our study, suggest that turtles from the Western Pacific region tend to temporarily inhabit this FG during their early juvenile stage, while turtles from Japanese populations might reside there for extended periods. Similar variations in population composition by size have also been observed in an FG off the coast of Australia in the southern Pacific, indicating the possible mixture of populations that undergo developmental migration and those that do not (Jensen et al. 2016b). More supporting evidence, including information on the developmental migration of Western Pacific populations, is needed to better understand the differences in habitat use among regional populations. Furthermore, factors other than migration patterns among natal populations need to be considered as well. For example, recent climate change has altered the distribution of many marine species (Doney et al. 2012, Poloczanska et al. 2016). Ectothermic marine turtles could change their habitat depending on the sea water temperature as an adaptation. Additionally, changes in ocean currents have the potential to influence the dispersal routes of post-hatchlings, leading to shifts in their distribution. These turtles might also adjust their distribution in response to the changes in the availability and distribution of food resources (Patrício et al. 2021).

4.3. Variation in the sex ratio between size classes

A recent study by Kameda et al. (2019) found that the female ratios in the Yaeyama FG decreased in both size classes. The <55 cm SCL size class showed a decrease from 76.4% (1997–1999) to 74.6% (2016–2017), while the ≥55 cm SCL size class showed a decrease from 55.4% (1997–1999) to 55.0% (2016–2017). In this study, we observed that turtles from the Ryukyus had the lowest female ratios in both size classes. This suggests a likely connection between the trend reported by Kameda et al. (2019) and an increased proportion of turtles originating from the Ryukyus in this FG. Interestingly, the female ratio in the turtles from the Western Pacific populations tended to be lower in the <55 cm SCL class than in the ≥55 cm SCL class, while those from Japanese populations exhibited the opposite trend.

The impacts of recent global warming on green turtle populations have led to feminization, particularly in the northern Great Barrier Reef (GBR) at low latitudes (Jensen et al. 2018). This feminization is attributed to temperature-dependent sex determination, where higher temperatures during embryo development result in more female turtles. In the GBR, the degree of feminization varies along latitudes, with a more pronounced effect observed in juveniles from rookeries at lower latitudes compared to higher latitudes (Jensen et al. 2018). However, our findings contradict the results reported by Jensen et al. (2018). We observed increased female ratios only in the smaller size class of individuals from high-latitude Japanese populations.

Another major concern related to feminization is the relocation of clutches to unshaded beach hatcheries for incubation (Jensen et al. 2016a, Reboul et al. 2021). However, this practice is rarely implemented in both the Ryukyus and the Ogasawara Islands. The nesting beaches in Ogasawara are characterized by abundant vegetation and environmental diversity, which can potentially influence nest temperatures (Kobayashi et al. 2020). The same is true for the Ryukyus. Female green turtles tend to choose nesting sites close to vegetation or at altitudes with low surface temperatures (Kelly et al. 2017, Patrício et al. 2018, Heredero Saura et al. 2022). It is likely that they exhibit similar nesting preferences in the Ryukyus and Ogasawara. Therefore, it is unlikely that extreme feminization through natural hatching has

occurred in Japan over the past 20 yr. Consequently, it is important to investigate factors other than feminization progression in rookeries to explain the high female ratios observed in the turtles from the Ryukyu and Ogasawara populations in the <55 cm SCL class.

4.4. Caveats of group assignment based on limited SNPs

In this study, we utilized genome-wide SNPs introduced by Hamabata et al. (2020) to estimate the natal origin of each individual within the Yaeyama foraging aggregation. We observed some inconsistencies between the sampled locality and the estimated natal origin when analyzing samples with known origins. This suggests that a similar mismatch might have occurred when estimating the origins of samples with unknown origins. The discrepancy could be attributed to both an inadequate number of samples with known origins and the population structure of green turtles in the northwestern Pacific.

Genetic analysis using mtDNA revealed that there is no significant differentiation of haplotype frequencies between certain populations in the Central Ryukyus and Ogasawara, suggesting a recent shift in nest sites of females from Ogasawara to the Ryukyus (Hamabata et al. 2014). Population structures analyzed through SNP analysis using MIG-seq supported a partial connection between the central Ryukyus and Ogasawara, as well as a continuous genetic structure within the Ryukyus. There were also partial connections observed between the Ryukyus and Western Pacific populations (Hamabata et al. 2020). The DAPC in our study indicated that many individuals from the Western Pacific and Ryukyus populations showed admixture. One individual from the Ryukyus exhibited admixture with Ogasawara (Fig. 3b; Fig. S5b). These complex population relationships in the northwestern Pacific contributed to the presence of mismatched samples in our study, making it challenging to accurately estimate the natal origin of individuals.

Marine turtles exhibit philopatry to their natal region, not only among females but also among males, and gene flow between regionally distant populations is rare (Roden et al. 2013). However, gene flow actually occurs between neighboring populations in the western North Pacific (Nishizawa et al. 2011) and might also occur over a wider region, as suggested in the GBR (FitzSimmons et al. 1997). Although mtDNA haplotypes can effectively differentiate certain regional populations, the ability to differentiate between individuals from Ogasawara and the Ryukyus using common Japanese haplotypes is limited. However, group assignments based on SNP analysis can distinguish them with an accuracy of 85% or higher. Therefore, this method proves to be more useful in understanding the demography of foraging areas predominantly occupied by individuals from Japanese rookeries.

Furthermore, although we utilized 1676 SNPs in our study, the use of higher-output sequencers can generate a larger number of SNPs, enabling a more precise estimation of the natal origin in this region. In the field of population genetics, with the availability of a highly accurate reference genome for green turtles assembled at the chromosome level, whole-genome analysis becomes feasible without limiting the analysis to specific loci. Cost-effective methods such as low-coverage whole-genome sequencing are emerging as powerful approaches for population genomic studies, even in non-model species (Lou et al. 2021). Future studies employing such methods are likely to enhance population resolution, even in regions characterized by complex genetic structures.

Acknowledgements. All samples analyzed in this study were collected in previous studies with permission from the Marine Fisheries Coordinating Committee of Okinawa Prefecture, Japan. Laparoscopic sex identification of those turtles was supported by Dr. Jun Noda, Dr. Kazuya Matsuda, and Rakuno Gakuen University during 2016–2018. We also thank the anonymous reviewers for their very helpful suggestions. This work was supported by JSPS grant-in-aid 18J00353 and KAKENHI Grant Number 21K15162. Computations were partially performed on the NIG supercomputer at ROIS National Institute of Genetics.

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Editorial responsibility: Per Palsbøll, Groningen, The Netherlands
Reviewed by: 4 anonymous referees

Submitted: July 19, 2022
Accepted: June 26, 2023
Proofs received from author(s): August 7, 2023