INTRODUCTION

Several marine vertebrates have complex life cycles, including the use of different habitats, some of which require long-distance dispersal (Boyle et al. 2009, Hays & Scott 2013). Successful dispersal is especially critical for endangered species, in which threats may be highly localized and yet have a potentially profound effect over distant areas. Therefore, understanding the dispersal pathway and connectivity of an endangered species will assist in the development of strategies for the effective conservation and management of specific marine organisms (Boyle et al. 2009, Carreras et al. 2013).

Among marine vertebrates, 6 out of 7 sea turtle species are listed as Endangered (IUCN 2013), and their long-distance dispersals have been reported. Loggerhead turtles Caretta caretta migrate throughout the
Pacific from Japan to Baja California, Mexico (Bowen et al. 1995); from the southern USA and Mexico to the Azores and Madeira in the Atlantic (Bolten et al. 1998); and from Australia in the southwestern Pacific to the coast of South America (Boyle et al. 2009). However, the dispersal distance of green turtles *Chelonia mydas* involves regional variation. Amorocho et al. (2012) suggested that the distant Western and Central Pacific nesting green turtle population contributed to the Gorgona green turtle feeding aggregation in the southeastern Pacific, whereas Dutton et al. (2008) estimated that the Hawaiian nesting population used the Hawaiian feeding area. In the Atlantic, the Ascension nesting population of green turtles was shown to contribute to the southwestern feeding aggregations thousands of kilometers away (Proietti et al. 2012, Prosdocimi et al. 2012), whereas green turtles born in West Africa were considered to be concentrated in the tropical eastern Atlantic (Godley et al. 2010). Reportedly, the dispersal patterns are influenced by oceanic currents, because the hatchlings engage mostly in passive pelagic drifting that may last several years after hatching in terrestrial habitats followed by recruitment to neritic habitats such as feeding areas (Musick & Limpus 1997, Reich et al. 2007).

Little is known, however, about the westward dispersal of hatchlings or young juveniles from the Hawaiian and eastern Pacific rookeries. Green turtles nesting mainly in Mexico and the Galapagos have a different carapace shape and coloration from those typical for the species and are thus sometimes considered a subspecies of green turtle called the ‘black turtle’ *Chelonia mydas agassizii* (Pritchard 1999). Black turtles are found in the Mexican Pacific during all life stages, and the coastal waters of the eastern Pacific serve as an important feeding and developmental habitat (Koch et al. 2007, López-Castro et al. 2010); they are also found in the ocean south of Hawaii (Parker et al. 2011). Genetic analyses have indicated that the Hawaiian and eastern Pacific rookeries contribute to the Colombian Gorgona feeding aggregation in the eastern Pacific (Amorocho et al. 2012) and that the Hawaiian feeding aggregation of green turtles mostly originated from a Hawaiian nesting population (Dutton et al. 2008). Although the findings indicate that the green turtles born in Hawaiian and eastern Pacific rookeries remain in Hawaii and the eastern Pacific Ocean, there are some anecdotal reports of black turtles in Japanese coastal areas (Abe & Minami 2008, Hayashi et al. 2011, Nishizawa et al. 2013), which is suggestive of westward long-distance dispersal. However, because only a few specimens of black turtles in Japanese coastal areas have been found and examined, genetic support of this westward long-distance dispersal is lacking.

The dispersal pattern of green turtles to the northwestern Pacific has recently been estimated from the genetic composition of the Japanese feeding aggregations. The southern feeding aggregation around Yaeyama was reported to originate from various Pacific rookeries, including Yaeyama, Ogasawara, and tropical Pacific regions, but the contribution from the tropical Pacific rookeries to the northern feeding aggregation is decreasing, and there is a significant correlation between genetic and geographical distance matrices of feeding aggregations (Nishizawa et al. 2013). The results of the latter study indicated that most hatchlings from these regions, which are transported by the Kuroshio Current (Fig. 1), settle in upstream feeding areas along the Japanese Archipelago (Nishizawa et al. 2013). A previous study on Japanese feeding aggregations of green turtles failed to estimate substantial westward dispersal from the Hawaiian and eastern Pacific rookeries, probably because of the rarity of individuals from the rookeries (Nishizawa et al. 2013). However, the genetic composition of species inhabiting the northernmost feeding area of Japan, the Sanriku coast, where the black turtle was found (Hayashi et al. 2011), has not yet been established. The Sanriku coast off the Japanese Archipelago is one of the most productive fishery regions in Japan (Sugimoto & Tameishi 1992) and serves as the northern edge of sea turtle feeding areas (Fig. 1; Narazaki 2009). To further confirm the dispersal of the Pacific green turtle, determining its genetic composition in the northernmost feeding area is warranted.

In this study, we investigated the genetic composition of green turtles in the Sanriku feeding area and estimated the contribution of the Pacific rookeries and the dispersal pattern by using mixed-stock analysis (MSA) (Pella & Masuda 2001, Bolker et al. 2003, 2007). The specific issues we addressed were: (1) whether the tendency of change in composition from south to north can be applied to this feeding aggregation and (2) whether there are any westward long-distance dispersals in the Pacific Ocean, especially from the Hawaiian and eastern Pacific rookeries.

### MATERIALS AND METHODS

Samples were collected from 39 green turtles by-caught in set nets at the Sanriku coast in Japan (Otsuchi Bay between Miyako and Ofunato) (38° 55’ to 39° 40’ N, 141° 40’ to 142° 05’ E; Fig. 1). Minute pieces of tissue (ca. 5 mm in diameter), which were
sampled while punching flippers for tagging, were collected and stored in 99% ethanol. Samples were collected in 2007 (n = 8), 2008 (n = 9), 2009 (n = 5), 2010 (n = 4), and 2012 (n = 13). Straight carapace length (SCL) of the turtles ranged from 38.9 to 85.6 cm. One of the specimens in 2009 was identified as a black turtle based on phenotypic features (Hayashi et al. 2011), including dark plastral coloration and marked incurving of the posterolateral shell margin above each hind flipper (Fig. 2). In addition, 2 other black turtles were collected as bycatch in set nets in the Sanriku coastal area in 2010 (tissues were not collected) (Table 1).

DNA extraction, polymerase chain reaction (PCR), and sequencing reaction followed the methodology of Nishizawa et al. (2014). DNA was extracted from a small amount of tissue and prepared for PCR using the Blood and Tissue Genomic DNA Miniprep System (Viogene). An 814 bp segment of the mtDNA control region was PCR amplified using the primers LCM15382 (5’-GCT TAA CCC TAA AGC ATT GGG-3’) and H950g (5’-GTC TCG GAT TTA GGG GTT TG-3’) (Abreu-Grobois et al. 2006). Typically, a 3.0 µl template was used in a 20 µl PCR reaction containing 2.0 µl 10× PCR buffer, 2.0 µl deoxynucleoside triphosphates (dNTPs) (2 mM), 0.8 µl magnesium sulfate (25 mM), 1.5 µl forward and reverse primers (2.0 µM), and 0.4 µl KOD-Plus (Toyobo) under the
following conditions: 2 min at 94°C, followed by 35 cycles of 15 s at 94°C, 30 s at 58°C, and 20 s at 68°C. Alternatively, a 1.0 to 2.5 µl template was used in a 15 µl PCR reaction containing 1.5 µl 10× PCR buffer, 1.2 µl dNTPs (2 mM), 0.6 µl forward and reverse primers (2.0 µM), 0.2 µl bovine serum albumin, and 1.2 µl PCR product (1.0 to 1.8 µl depending on the results of PCR) was purified using ExoSAP-IT (GE Healthcare Bio-Sciences KK).

Sequencing reactions (forward and reverse) were performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Cycle sequencing was done in 5 µl reactions with typically 1.5 µl PCR product (1.0 to 1.8 µl depending on the results of electrophoresis), 1.0 µl 5× sequencing buffer, 1.2 µl 2.0 µM primer, 0.8 µl sterilized water, and 0.5 µl dye terminator, with 1 min at 96°C, followed by 30 cycles of denaturing at 96°C for 10 s, annealing at 56°C for 5 s, and extension at 60°C for 1 min. Products were purified with ethanol precipitation and run through a 3130xl sequencing analyzer (Applied Biosystems).

Sequence alignments were performed using CLUSTALW in MEGA v5.1 (Tamura et al. 2011). An unrooted haplotype network was created using TCS v1.21 (Clement et al. 2000), and nucleotide diversity (θ) and haplotype diversity (h) were estimated using ARLEQUIN v3.5 (Excoffier & Lischer 2010). Comparisons of genetic composition of the Sanriku feeding aggregation with other aggregations along the Japanese Archipelago, namely Yaeyama, Ginoza, Nomaikc, Muroto, and Kanto (Hamabata et al. 2009, Nishizawa et al. 2013), were conducted using exact tests (500 000 simulations, which was designed for control region sequences (Tamura & Nei 1993).

The Bayesian MSA was used to estimate relative contributions of nesting source populations to the Sanriku feeding aggregation. The methodology of MSA followed Nishizawa et al. (2013). Briefly, we compared 2 MSA methods to check the robustness of the estimates: the many-to-one Bayesian MSA using BAYES (Pella & Masuda 2001) and the many-to-many MSA (Bolker et al. 2007). The latter analysis included reanalysis of Yaeyama, Ginoza, Nomaikc, Muroto, and Kanto feeding aggregations (Hamabata et al. 2009, Nishizawa et al. 2013). The source populations included a total of 25 geographically or genetically separated rookeries in the Pacific and eastern Indian Ocean regions, including the Japanese rookeries of Yaeyama (Nishizawa et al. 2011) and Ogasawara (Nishizawa et al. 2013), Taiwan rookeries (Cheng et al. 2008), the eastern Pacific rookeries of Mexico (Chassin-Noria et al. 2004), Hawaii, the Galapagos (Dutton et al. 2008), and 17 Australasian rookeries as defined by Dethmers et al. (2006). These populations were classified into 6 regions: Yaeyama, Ogasawara, Taiwan, Hawaii and eastern Pacific, western Pacific, and eastern Indian Ocean and Southeast Asia (see Fig. 4 and Nishizawa et al. 2013). Both Bayesian analyses used Markov Chain Monte Carlo (MCMC) to simulate unknowns from the posterior distribution. Six MCMC chains of 50 000 samples were run, with each chain corresponding to a potentially contributing nesting group. The first 25 000 samples of each chain were discarded as burn-in to remove dependence on starting values. Remaining samples were pooled and summarized. The convergence of MCMC sampling to
the posterior distribution was assessed using the Gel-
man-Rubin shrink factor (Gelman & Rubin 1992),
which provides an indication of convergence by com-
paring the variation within a single chain to the total
variation among all chains. Shrink-factor values >1.2
indicate lack of convergence in both Bayesian analy-
ses. If convergence was not achieved, we increased
the number of samples of each chain up to 100 000
with half burn-in steps. Individual chains were started
with 95% of the mixed sample initially contributed by
each group of source populations, and the remaining
5% was divided equally among the remaining popu-
lations. The Dirichlet prior distribution was set in 2
ways, as described by Nishizawa et al. (2013). One
was an uninformative prior giving all population pro-
portions equal weights, and the other was an informa-
tive prior considering the effect of distance and popu-
lation size based on Moritz et al. (2002), Dethmers et
al. (2006), and Amorocho et al. (2012). In the latter
case, the prior was weighted by the population size
multiplied by the inverse of the straight distance in
the many-to-one MSA, but by the inverse of the
straight distance and population size that was set sep-
arately in the program in the many-to-many analysis.
For comparison with feeding aggregations and
linking to nesting populations in the earliest studies
of green turtles (Chassin-Noria et al. 2004, Dethmers
et al. 2006, Nishizawa et al. 2013) in the above analy-
ses, the sequence datasets were truncated to include
only the 380 bp fragment of the control region when
necessary.

RESULTS

Screening for polymorphisms within the 814 bp
mtDNA control region fragment among 39 turtles
identified 9 haplotypes (Fig. 3, Table 2). Most
samples (30 of 39) showed haplotypes CmP39.1 or
CmP50.1, which were longer fragments of the haplo-
types previously detected only from Japanese rook-
eries, CMJ18 and CMJ25, respectively (Nishizawa et
al. 2011, 2013). CmP121.1 and CmP127.1 contained
sequences specific to Ogasawara rookeries in Japan,
CMJ15 and CMJ19, respectively (Nishizawa et al.
2013). CmP70.1 was detected in Ryukyus, Japan, and
registered as GenBank Accession no. AB819812 (Ha-
mabata et al. 2014). The CmP20.1 haplotype that was
detected from 1 sample was a longer fragment of

<table>
<thead>
<tr>
<th>Fragment (bp)</th>
<th>Haplotype</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>814</td>
<td>CmP39.1</td>
<td>CmP50.1</td>
</tr>
<tr>
<td>520</td>
<td>CMJ18</td>
<td>CMJ25</td>
</tr>
<tr>
<td>380</td>
<td>CmP39</td>
<td>CmP50</td>
</tr>
<tr>
<td>No. of samples</td>
<td>22</td>
<td>8</td>
</tr>
</tbody>
</table>
CmP20 that was previously reported from the western Pacific, and eastern Indian Ocean and Southeast Asian rookeries (Dethmers et al. 2006). The sample from the black turtle carried a CmP129.1 haplotype that did not match any previously reported sequences (Table 1) but contained a single base substitution within the 380 bp fragment of CmP5 that was detected in the Mexican rookery (Dutton et al. 2008). This haplotype was deposited in GenBank (Accession no. AB856322). In addition, 3 samples from specimens with typical green turtle phenotypes showed haplotypes CmP1.1 and CmP3.1 (Table 1), which include the sequences observed in the Hawaiian and eastern Pacific rookeries (Dutton et al. 2008).

$h$ and $\pi$ estimates (±SD) for the Sanriku feeding aggregation were $h = 0.6478 ± 0.0745$ and $\pi = 0.01434 ± 0.0074$. When the sequence was trimmed to about 380 bp for comparisons with the other Japanese feeding aggregations (Nishizawa et al. 2013), estimates were $h = 0.6478 ± 0.0745$ and $\pi = 0.023132 ± 0.012103$ (Table 3). Comparison of haplotype frequency among size classes showed no significant differences ($p > 0.05$). Therefore, all samples were pooled in the subsequent MSA. The haplotype frequency of the Sanriku feeding aggregation was significantly different from that of the Yaeyama ($p < 0.00001$) and Ginoza feeding aggregations ($p = 0.00584$), although the significance in the latter case disappeared after sequential Bonferroni correction (Table 4). The Mantel test revealed a significant correlation between genetic distances as measured by $\Phi_{ST}$ values and geographical distance measures ($r = 0.692; p = 0.0097$).

Many-to-one MSA estimations of 25 rookeries showed Gelman-Rubin shrink factors of 1.01 or lower, demonstrating that the Sanriku feeding area was extensively used by turtles originating mainly from Ogasawara. In addition, a small but substantial contribution (lower limit of 95% credible intervals >1%) from Hawaii to the Sanriku aggregation was detected, regardless of whether or not the informative prior was considered (Fig. 4). The Gelman-Rubin shrink factors for Bayesian estimates in the many-to-many MSA using 25 rookeries as the source indicated a lack of convergence, even when the samples of each chain were increased up to 100,000 (data not shown). Estimates in the many-to-many MSA using 6 groups of rookeries as the source showed Gelman-Rubin shrink factors of 1.05 or lower. Group estimates for both the many-to-one MSA and many-to-many MSA supported the substantial contribution (lower limit of 95% credible intervals >1%) of the Ogasawara and Hawaiian and eastern Pacific rookeries to the Sanriku feeding aggregation (Fig. 5). The results of the many-to-many MSA in the other feeding areas supported the findings of Nishizawa et al. (2013).

### DISCUSSION

Understanding the dispersal pathway and connectivity in an endangered species plays an essential role in the development of strategies for its effective conservation and management (Boyle et al. 2009, Carreras et al. 2013). Recently, investigations into mtDNA haplotype frequencies caused by genetic isolation among nesting populations have established the relationship between feeding aggregations and their rookery of origin as well as facilitated MSA estimations of the contributions of genetically differentiated nesting populations to foraging assemblages (Pella & Masuda 2001, Bolker et al. 2003). The MSA approach is useful in establishing the linkage between nesting populations and feeding aggregations and in detecting long-distance dispersals of marine vertebrates.
MSA indicated that the feeding aggregation in the Sanriku coastal area migrated mainly from Ogasawara and specifically from the Hawaiian and eastern Pacific rookeries. A recent report also identified the high contribution of the Ogasawara to the Nomazima, Muroto, and Kanto feeding aggregations (Nishizawa et al. 2013). The significant difference in haplotype frequency between the Sanriku and southern feeding aggregations of Yaeyama and Ginoza and the significant correlation between genetic and geo-
graphical distance measures in the Mantel test suggest that the tendency for compositional change from south to north can be applied to the Sanriku aggregation. Compositional change may be driven by hatchlings from nesting colonies in the tropical Pacific regions that were transported to Japanese coastal areas by the Kuroshio Current but did not settle in the northern feeding aggregations (Nishizawa et al. 2013).

Besides the compositional change as indicated by Nishizawa et al. (2013), the small but substantial contribution to the Sanriku coast from remote Hawaiian and eastern Pacific rookeries indicates the occurrence of the westward long-distance dispersal of hatchlings or young juvenile green turtles in the Pacific. The results reflect the detection of haplotypes specific to the Hawaiian and eastern Pacific rookeries, CmP1.1 and CmP3.1 (Dutton et al. 2008). CmP129.1 detected in the black turtle specimen did not match any of the previously reported sequences and was discarded from our MSA but was similar to the sequence of CmP5 that was detected in the Mexican rookery. These results genetically support the westward long-distance dispersal of green/black turtles as indicated by some anecdotal reports of black turtles in Japanese coastal areas (Abe & Minami 2008, Nishizawa et al. 2013). These results genetically support the westward long-distance dispersal of green/black turtles as indicated by some anecdotal reports of black turtles in Japanese coastal areas (Abe & Minami 2008, Nishizawa et al. 2013) and in Sanriku (see Table 1).

The westward dispersal observed in this study is in contrast to the reported eastward long-distance dispersal and dominance of green turtles born in Hawaii and the eastern Pacific Ocean for these feeding aggregations (Dutton et al. 2008, Amorocho et al. 2012). A concentration of green turtles born in the tropical eastern Atlantic rookeries in the waters around the rookeries has also been estimated (Godley et al. 2010). The westward dispersal of hatchling or young juvenile turtles has been indicated for southern Atlantic green turtles (Proietti et al. 2012, Prosocimini et al. 2012), but to our knowledge, the present study is the first that genetically supports the occurrence of the westward long-distance dispersal in the Pacific.

Westward long-distance dispersal in the Pacific indicates the complexity of green turtle migration, possibly reflecting variations in the geographic distribution of their nesting places. While the Hawaiian and eastern Pacific feeding aggregations of green turtles originated mostly from Hawaiian and eastern Pacific nesting populations (Dutton et al. 2008, Amorocho et al. 2012), the present study indicates that these nesting populations also contribute to the Sanriku feeding aggregation. The North Equatorial Current flowing westward can transport hatchlings originating from the Hawaiian and eastern Pacific rookeries to the west (Kessler 2006), possibly reaching the Japanese coast. The estimated contributions to feeding aggregations in our MSA should be carefully interpreted. The relatively small number of samples and pooled mixture samples over several years can normalize the temporal variations in the estimated contributions of the rookeries. In addition, the fact that haplotypes CmP70 and CmP129 were not matched to haplotypes detected from candidate nesting populations and so not included in our MSA indicates that other rookeries also contributed to the feeding aggregations investigated. Further studies might be needed to determine whether more green/black turtles from Hawaiian and eastern Pacific rookeries utilize the Sanriku coast than other Japanese coastal areas or whether the contribution from the rookeries to other Japanese areas is merely masked by that of other rookeries. Nonetheless, the results of this study provide evidence of westward long-distance dispersal of green turtles in the Pacific, in addition to changes in the genetic composition of green turtles from south to north along the Japanese Archipelago as indicated by Nishizawa et al. (2013).

Acknowledgements. This study could not have been completed without the assistance of volunteers from the Fisheries Cooperative Association of Funakoshi Bay, Hirota Bay, Kamaishi Bay, Kamaishi-Tobu, Miyako, Ofunato, Okirai, Omoe, Otsuchi, Ryori, Sanriku-Yamada, Sasaki, Toni, and Yoshihama in collecting the turtles. We also thank Y. Fujiwara, S. Takuma, H. Murasaki, and K. Onitsuka for their assistance in the field. P. Dutton and M. Jensen helped us name the haplotypes according to the standardized nomenclature. We thank the 3 anonymous reviewers for their valuable comments on this manuscript. Field sampling was financially supported by a Grant-in-Aid for Science Research from the Japan Society for the Promotion of Science (JSPS) and a grant from Bio-Logging Science, University of Tokyo (UTBLS). This study was partly supported by a Grant-in-Aid for JSPS Fellows to H.N. (25-1999).

LITERATURE CITED


Abreu-Grobois FA, Horrocks J, Formia A, Leroux R and others (2006) New d-loop primers which work for a variety of marine turtle species may increase the resolution capacity of mixed stock analyses. Presentation at the 26th Annu Symp Sea Turtle Biol Conserv, Crete, Greece, 2–8 April


Submitted: October 24, 2013; Accepted: February 19, 2014
Proofs received from author(s): May 14, 2014

Editorial responsibility: Jeffrey Seminoff,
La Jolla, California, USA