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Research Paper

Evidence of historical pairing between two cryptic species of Short-tailed Albatross

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ABSTRACT. When secondary contact occurs between allopatric sister species, several evolutionary consequences are expected, such as reinforcement of reproductive isolation, hybrid speciation, de-speciation, introgressive hybridization, or formation of a stable hybrid zone. The Short-tailed Albatross (*Phoebastria albatrus*) is a vulnerable seabird that breeds mainly in Torishima, the Izu Islands, and two islets in the Senkaku Islands in the western North Pacific. Recent studies revealed that Short-tailed Albatross comprises two cryptic species (Senkaku-type and Torishima-type) that breed sympatrically on Torishima. Ringed (hatched in Torishima) and unringed (probably hatched in the Senkaku Islands) birds mate in a mutually assortative manner at the Hatsunezaki colony (artificially established in 1995) on Torishima. However, observations of some ringed–unringed pairs suggest possible hybridization between the two cryptic species. To clarify the degree of hybridization, we analyzed microsatellite DNA and mitochondrial DNA control region 2 (CR2) sequences of chicks from Hatsunezaki and Tsubamezaki (original colony discovered in 1951) colonies and of unringed birds from Hatsunezaki. In general, both CR2 sequences and microsatellites revealed genetic differentiation between immigrants from the Senkaku Islands (unringed birds) and chicks hatched in Tsubamezaki. These findings support the existence of two cryptic species. Each chick obtained from four ringed–unringed parent pairs at Hatsunezaki displayed a high proportion of alleles from just a single population. In contrast, some chicks in Tsubamezaki had a medium proportion of alleles from both populations. Breeding unringed subadult plumage birds, which were probable immigrants from the Senkaku Islands, were observed in Hatsunezaki but not in Tsubamezaki. Therefore, we propose that interspecific pairing occurred in the past but infrequently in recent generations on Torishima, suggesting historical reinforcement of reproductive isolation. Further microsatellite DNA studies of chicks from Hatsunezaki are required to confirm whether reinforcement of reproductive isolation is achieved. Alternatively, nearly complete pre-mating isolation between the two species was established in the past, but the scarcity of Senkaku-type birds in Torishima has facilitated hybridization.

Des preuves d'accouplement historiques entre deux espèces cryptiques d'albatros à queue courte

RÉSUMÉ. Lorsqu'un contact secondaire se produit entre des espèces allopatriques sœurs, plusieurs conséquences sont à prévoir sur le plan de l'évolution, notamment un renforcement de l'isolement reproductif, une spéciation par hybridation, une déspéciation, une hybridation introgressive ou la formation d'une zone hybride stable. L'albatros à queue courte (*Phoebastria albatrus*) est un oiseau marin vulnérable qui se reproduit principalement à Torishima, dans les îles d'Izu et sur deux îlots des îles Senkaku, dans la région ouest du nord Pacifique. Des études récentes ont révélé qu'il existe deux espèces cryptiques d'albatros à queue courte (le type Senkaku et le type Torishima) qui se reproduisent de manière sympatrique à Torishima. Les oiseaux annelés (nés à Torishima) et non annelés (probablement éclos dans les îles Senkaku) se reproduisent de manière mutuellement assortative dans la colonie d'Hatsunezaki (un lieu établi artificiellement en 1995) à Torishima. Toutefois, des observations de couples annelés-non annelés suggèrent une hybridation possible entre les deux espèces cryptiques. Pour clarifier le degré d'hybridation, nous avons analysé des séquences d'ADN de microsatellites et d'ADN mitochondrial de la région de contrôle 2 (CR2) chez des oisillons des colonies d'Hatsunezaki et de Tsubamezaki (colonie originale découverte en 1951) et d'oiseaux non annelés provenant d'Hatsunezaki. D'une manière générale, les séquences de la CR2 et des microsatellites ont révélé une différenciation génétique entre les oiseaux des îles Senkaku (non annelés) et les oisillons nés à Tsubamezaki. Ces découvertes confirment l'existence de deux espèces cryptiques. Chaque oisillon né de quatre paires de parents annelés-non annelés à Hatsunezaki présentait une forte proportion d'allèles issus d'une seule population. En revanche, certains oisillons de Tsubamezaki possédaient une proportion moyenne d'allèles provenant des deux populations. La reproduction d'oiseaux à plumage subadulte non annelé, probablement immigrés des îles Senkaku, a été observée à Hatsunezaki, mais pas à Tsubamezaki. En conséquence, nous pensons que des accouplements interspécifiques se sont produits dans le passé, mais peu fréquemment dans les générations récentes à Torishima, ce qui suggère un renforcement historique de l'isolement reproductif. D'autres études de microsatellites d'ADN d'oisillons provenant d'Hatsunezaki seront nécessaires pour confirmer l'existence ou non d'un isolement reproductif. D'autre part, un isolement pré-accouplement quasi-complet entre les deux espèces a été établi dans le passé, mais la rareté des oiseaux de type Senkaku à Torishima a favorisé l'hybridation.

Key Words: *conservation; Diomedeidae; hybridization; reinforcement; reproductive isolation*

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INTRODUCTION

Hybridization, the interbreeding of species, has been reported in ~10% of bird species (Grant and Grant 1992) and is thought to occur unrecognized in further species (Grant and Grant 1992, McCarthy 2006). When secondary contact occurs between allopatric sister species, expected evolutionary consequences include reinforcement of reproductive isolation, formation of a new species (hybrid speciation), fusion or genetic swamping of one species (de-speciation), transfer of genetic components between the species (introgressive hybridization), or formation of a stable hybrid zone (Seehausen 2004, Jacobsen and Omland 2011, Hasegawa 2012, Brown et al. 2015, Grant and Grant 2019). If the sister species are conservation targets, it is important to estimate the evolutionary consequences of secondary contact to prioritize the target species and area and establish efficient conservation strategies.

The Short-tailed Albatross (*Phoebastria albatrus*) is a vulnerable seabird species that breeds primarily in Torishima, the Izu Islands, and two islets in the Senkaku Islands in the western North Pacific (U.S. Fish and Wildlife Service 2008, BirdLife International 2018). In the late 19th century, several million birds bred in at least 13 breeding colonies; however, their numbers have been drastically reduced owing to feather hunting on these breeding islands (Tickell 2000, Hasegawa 2003, U.S. Fish and Wildlife Service 2008). Since feather hunting ceased, the number of birds has increased with the aid of conservation efforts (Sato 2009, Hasegawa 2015, Deguchi et al. 2017). At the end of the 2013/2014 breeding season, the estimated global Short-tailed Albatross population was 4200 individuals, including 3540 originating from Torishima Island and at least 650 from two islets in the Senkaku Islands, and 10 birds on Ogasawara Islands (BirdLife International 2018).

The Short-tailed Albatross is tacitly regarded as a single management unit, and international conservation organizations have not considered its population structure. However, based on morphological, genetic, and ecological differences, it was recently revealed that the Short-tailed Albatross comprises two cryptic species (Senkaku-type and Torishima-type; Eda et al. 2020). The scientific species name *Phoebastria albatrus* should be applied only to the Senkaku-type, whereas the scientific name of the Torishima-type is undetermined (Yamasaki et al. 2022). Torishima-type birds are generally larger than Senkaku-type birds, while the latter have relatively longer beaks (Eda et al. 2020). The sequence divergence of the mitochondrial DNA (mtDNA) cytochrome *b* region between the two types of bird is greater than differences between other Diomedeidae sister species (Eda et al. 2011, Eda and Higuchi 2012), and birds from the Senkaku Islands and Torishima show largely mutual monophyly in mtDNA control region 2 (CR2) sequences (Kuro-o et al. 2010, Eda et al. 2012). Furthermore, ancient DNA, stable isotope, and morphometric analyses of zooarchaeological bones reveal that birds from different CR2 clades formed different populations ~1000 years ago (Eda et al. 2012).

Ecological differences between the two cryptic species include differences in courtship displays (F.S., *personal observation*) and in the breeding season, which occurs ~2 weeks earlier on the Senkaku Islands than on Torishima (Kuroiwa 1900, Miyajima 1900, Hasegawa 2006). In addition, mutual assortative mating of the two cryptic species is suggested in Torishima. There are two colonies of the species in Torishima: Tsubamezaki and Hatsunezaki. The Tsubamezaki colony was discovered in 1951 as the original colony, while the Hatsunezaki colony was artificially established in 1995 by attracting birds using decoys and audio devices (Sato 2009). Despite the morphological difference between the two cryptic species, it is difficult to discriminate between them accurately using a telescope from a long distance. On Torishima, almost all birds that have hatched since 1979 have been ringed on at least one leg (Sato 1999, Yamashina Institute for Ornithology 2005). However, unringed birds with subadult plumage have been observed in Torishima, especially at Hatsunezaki, every year since 1996, and the number of unringed birds has increased in recent years (Sato 1999, Eda et al. 2016, 2020). Because it is unlikely that these unringed birds hatched on Torishima before 1979 or that they lost their ring(s), the birds are considered to be immigrants from the Senkaku Islands. Pairing of these unringed young birds was observed in Hatsunezaki but not in Tsubamezaki (F.S., *personal observation*). In a census study, ringed (91.5% of observed birds) and unringed birds (8.5%) showed a clear trend of assortative mating in Hatsunezaki, where observed outbreeding, i.e., ringed-unringed pairs (6.3%), were significantly fewer than expected outbreeding pairs (15.6%), whereas the existence of outbreeding pairs suggests incomplete pre-mating isolation (Eda et al. 2016). However, the observed outbreeding rate may be overestimated since the exact lineage of birds on Torishima is unknown. All the analyzed samples from the Senkaku Islands, and unringed birds on Torishima, together with approximately 7% of the chicks hatched at Tsubamezaki, belonged to a particular mtDNA CR2 sequence clade, clade A (Kuro-o et al. 2010, Eda et al. 2011, 2012, 2016, 2020). Therefore, some ringed birds mating with unringed birds may be descendants of birds that emigrated from the Senkaku Islands and bred on Torishima.

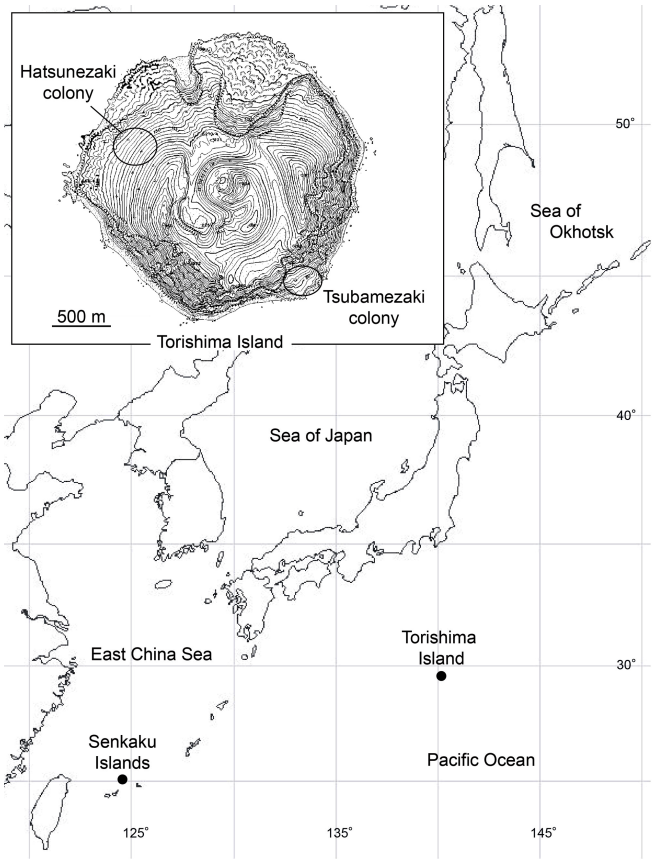
In this study, we analyzed mtDNA CR2 and microsatellite DNA of unringed birds and their chicks that hatched at Hatsunezaki, as well as chicks that hatched at Tsubamezaki. The aims of this study were to (1) examine whether the existence of two cryptic species was supported by nuclear DNA analysis, (2) clarify the degree of hybridization between the two cryptic species in the three groups, and (3) estimate the evolutionary consequences of secondary contact between the two cryptic species.

MATERIALS AND METHODS

We collected blood samples from Short-tailed Albatrosses captured at Tsubamezaki, Torishima (30.4763°N, 140.3090°E), during the 2007/2008 breeding season and at Hatsunezaki, Torishima (30.4862°N, 140.2919°E), during the 2012/2013 and 2013/2014 breeding seasons (Fig. 1). In Tsubamezaki, we captured 39 chicks that hatched during the breeding season. In Hatsunezaki, we captured two kinds of birds: (1) unringed birds (n = 9) and (2) chicks of which at least one parent was unringed (n = 13). Of the nine captured unringed birds, seven bred at the colony during the breeding season (Appendix 1); five of them mated with unringed birds, one mated with a ringed bird, and one mated with a bird of unknown ring status. Chicks were obtained from eight nests: four from unringed-unringed (UU) pairs and four from ringed-unringed pairs. One unringed bird from the ringed-unringed pairs was a male albatross, named "Deko-chan,"

which was observed after 1996 as the first immigrant from the Senkaku Islands to Hatsunezaki and made a nest close to decoy No. 22 during the 1997/1998 and 2013/2014 breeding seasons (Sato 1999). Genetic analysis revealed that Deko-chan had a mtDNA CR2 sequence belonging to clade A (Eda et al. 2011). The captured chicks included five sibling pairs born in different years. Five chicks, including two sibling pairs, were offspring of captured unringed birds. To minimize disturbance to the breeding behavior of birds in the colony, we carefully selected isolated individuals as capture targets. Blood samples (~0.5 ml) were taken from the cutaneous ulnar vein of birds. Blood samples were preserved in 99.5% ethanol and stored, first in a cool dark place (in the field) and then at 4 °C (in the laboratory), until analysis. The capture of Short-tailed Albatrosses was conducted with permission from the Ministry of the Environment, Government of Japan and the Agency for Cultural Affairs, Government of Japan, and in compliance with their guidelines.

Fig. 1. Locations of the Short-tailed Albatross (*Phoebastria albatrus*) breeding sites.



DNA analysis

Whole DNA was extracted from samples of blood and feathers using a Gentra Puregene Tissue kit (QIAGEN, Venlo, Netherlands) and a QIAamp DNA Mini kit (QIAGEN). Each sampled bird was genotyped at 10 polymorphic microsatellite loci, and the partial CR2 sequence was obtained.

MtDNA analysis

A partial sequence of CR2 domain I (341 bp) was amplified using the primers Lcon2.dio (Eda et al. 2010) and H454.gr (Baba et al. 2005). The target region sequences for chicks from Tsubamezaki and Hatsunezaki were determined in this study, because the sequences (and sex) for the nine unringed birds captured at Hatsunezaki were analyzed and published in Eda et al. (2016, 2020). Polymerase chain reaction (PCR) was performed using Ex *Taq* polymerase (Takara, Kusatsu, Japan) with the following conditions: an initial denaturation at 94 °C for 2 min; 40 cycles of 30 s at 94 °C, 45 s at 60 °C, and 2 min at 72 °C; and a final 5-min extension at 72 °C. The PCR products were cycle-sequenced using an ABI BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and run on an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems). The obtained sequences were aligned with unique sequences from *P. albatrus* individuals sampled on Torishima and the Senkaku Islands (DDBJ/EMBL/GenBank accession numbers AB254197-AB254200, AB254205-AB254206, AB254211, AB254217-AB254222, AB254226-AB254228, AB254230, AB254232, AB254234-AB254235, AB254238, LC066675-LC066677, and LC534780-LC534782) using ClustalW in MEGA X (Kumar et al. 2018).

The best-fit nucleotide substitution model was selected using the Bayesian information criterion, and a maximum likelihood (ML) tree was constructed with 1000 bootstrap replications using MEGA. Four sequences of Laysan Albatross (*P. immutabilis*; AB276048-AB276050 and AB276055) and five sequences of Black-footed Albatross (*P. nigripes*; AB276051, AB276057, AB276059, AB276061, and AB276063) were included as outgroup sequences.

Microsatellite DNA analysis

Primers isolated from Wandering Albatross *Diomedea exulans* were used: loci Dc5, Dc9, Dc20, and De11 from Burg (1999) and loci 10C5, 11F3, 11H1, 11H7, 12C8, and 12H8 from Dubois et al. (2005). Each forward primer was fluorescence labeled at the 5′ end with 6-FAM, VIC, PET, or NED (Applied Biosystems). PCR was performed using Ex *Taq* polymerase (Takara). The cycling profile was 1 cycle of 3 min at 94 °C; 35 cycles of 30 s at 94 °C, 30 s at 57 °C (Dc5, Dc9, De11 10C5, 11F3, 11H1, 11H7, 12C8, and 12H8) or 60 °C (Dc20), and 35 s at 72 °C; and a final extension of 5 min at 72 °C. The PCR product sizes were measured using the ABI 3730 DNA Analyzer with the GeneScan 600LIZ size standard and GeneMapper analysis software (Applied Biosystems).

The observed number of alleles per locus (A), number of effective alleles (N_E), and expected (H_E) and observed heterozygosity (H_O) were calculated using GENALEX 6.502 (Peakall and Smouse 2012). The allelic richness (A_R) and inbreeding coefficient (F_{IS}) were calculated using FSTAT 2.9.3 (Goudet 2001). FSTAT was also used for tests of the deviation of F_{IS} values from zero. Deviation from linkage equilibrium for all pairs of loci in each group was tested using Genepop 4.7.5 (Rousset 2008). Because eight of the chick samples at the Hatsunezaki colony were offspring of sampled unringed birds or one of the siblings, they were excluded from these analyses. We performed individual-based clustering analysis using the STRUCTURE 2.3.4 software (Pritchard et al. 2000). We used the admixture model and allele

frequency correlated models. The appropriate number of genetic populations (K) and the proportion of alleles that originated from population K ($q_k^{(i)}$) was estimated. Ten runs of K = 1-5 were carried out with 1,000,000 Markov chain Monte Carlo (MCMC) iterations, with the first 100,000 samples discarded as burn-in. STRUCTURE HARVESTER 0.6.94 (Earl and vonHoldt 2012) was used to determine the optimum K value using the delta K statistic (Evanno et al. 2005). The 10 independent runs with the best K values were processed using CLUMPP 1.1.2 (Jakobsson and Rosenberg 2007). Eight of the chick samples from the Hatsunezaki colony were also excluded from the STRUCTURE analysis. On the other hand, to check the possibility of extra-pair mating, the alleles of each parent-chick pair were examined to see if they shared at least one allele, and additional STRUCTURE analysis was conducted including the eight chicks.

RESULTS

MtDNA analysis

The target mtDNA CR2 sequence was determined for 39 chicks from Tsubamezaki and 13 chicks from Hatsunezaki. Because an overlapping peak (C and T) at the 77th site in sequence electropherograms of TC08-25 and TC08-27 was consistently found, nucleotide information of the site was excluded from the following analysis. A total of 17 distinct sequences were obtained. Comparison of the obtained sequences with those available in the DDBJ/EMBL/GenBank databases revealed that one sequence was newly observed, which was deposited into the databases with accession number LC534788. The HKY + G model was selected as the best-fit nucleotide substitution model based on the Bayesian information criterion. The ML tree showed two major clades in the Short-tailed Albatross: clades A and B were supported by 89% and 47% bootstrap values, respectively (Fig. 2). Clade A consisted of six sequences from 18 individuals, including all nine unringed birds and seven chicks from Hatsunezaki, and two chicks from Tsubamezaki. Clade B consisted of 11 sequences from 43 individuals, including 6 and 37 chicks, respectively, from Hatsunezaki and Tsubamezaki. Two sequences from clade A (A1 and A2) were shared between unringed birds and chicks from Hatsunezaki, while one sequence from clade A (A4) and four sequences from clade B (B1, B2, B8, and B11) were shared between chicks from Hatsunezaki and Tsubamezaki. No sequence was shared between unringed birds from Hatsunezaki and chicks from Tsubamezaki. All sibling pairs (five cases) had the same sequence, whereas one mother-chick pair had the same sequence (Appendix 1). A chick from Deko-chan's nest had the CR2 sequence type B1.

Microsatellite DNA analysis

The estimators of genetic diversity in each group (A, N_E, A_R, H_O, H_E, and F_{IS}) are shown in Table 1 and Appendix 2. On average, the values of A, N_E, A_R, H_O, and H_E were higher in chicks from Tsubamezaki than in unringed birds and chicks from Hatsunezaki. F_{IS} values significantly larger than zero were found in chicks from Tsubamezaki (in Dc20, 12C8, and all loci combined) and Hatsunezaki (12C8 and all loci combined). Deviation from linkage equilibrium was observed for the locus pairs Tc65 and Dc5 ($p < 0.05$) and De11 and 12E1 ($p < 0.01$) in chicks from Tsubamezaki (Appendix 3).

Table 1. Overall genetic diversity (A: number of alleles per locus, N_e: number of effective alleles, A_R: allelic richness, H_E: expected heterozygosity, H_O: observed heterozygosity, F_{IS}: inbreeding coefficient) of the Short-tailed Albatross (*Phoebastria albatrus*) based on 10 microsatellite loci.

	A	N_e	A_R	H_E	H_O	F_{IS}
Chicks from Tsubamezaki (n = 39)	5.200	3.028	3.506	0.622	0.577	0.085**
Unringed birds from Hatsunezaki (n = 9)	3.700	2.357	3.006	0.451	0.467	0.025
Chicks from Hatsunezaki (n = 5)	3.100	2.366	3.100	0.506	0.400	0.313**

**: significant departure from Hardy-Weinberg equilibrium ($p < 0.01$).

STRUCTURE analysis inferred the optimal number of populations for the Short-tailed Albatross as K = 2 (Table 2). A high proportion ($q_{k(i)} \geq 0.85$) of alleles from population 1 was observed in eight of nine unringed birds, four of 39 chicks from Tsubamezaki, and four of five chicks from Hatsunezaki, whereas a high proportion of alleles from population 2 was observed in 23 chicks from Tsubamezaki and one chick from Hatsunezaki (Fig. 3, Appendix 1). One unringed bird and 12 chicks from Tsubamezaki had mixed ancestry. A chick obtained from Deko-chan's nest had a high proportion of alleles from population 1.

Table 2. Mean and standard deviation of LnP (K) and Delta K for each K (K = 1 to K = 5) estimated by STRUCTURE.

K	Mean LnP(K)	Stdev LnP(K)	Delta K
1	-1316.83	0.86	—
2	-1282.82	3.29	29.50
3	-1345.94	12.12	3.22
4	-1370.05	16.48	2.36
5	-1433.08	121.69	—

On combining the mtDNA and microsatellite data, birds with the clade A CR2 sequence and a high proportion of alleles from population 1 were observed in eight unringed birds, one chick from Tsubamezaki, and two chicks from Hatsunezaki, whereas those with the clade B CR2 sequence and a high proportion of alleles from population 2 were observed in 22 chicks from Tsubamezaki and one chick from Hatsunezaki (Fig. 3). One chick from Tsubamezaki had the clade A CR2 sequence but a high proportion of alleles from population 2, while three chicks from Tsubamezaki and two chicks from Hatsunezaki had the clade B CR2 sequence but a high proportion of alleles from population 1.

On examining allele sharing between the five parent-chick pairs from Hatsunezaki, it was confirmed that all pairs shared at least one allele (Appendix 4). In the additional STRUCTURE analysis focused on the sibling pair and parent-chick pair, the estimated proportion of alleles from each population was similar in most of the birds on comparison with the result of original STRUCTURE analysis (Appendix 1). A high proportion of alleles from population 1 was observed in six chicks, while a high proportion of alleles from population 2 was observed in one chick,

Fig. 2. Maximum likelihood tree of mitochondrial control region 2 sequence for the Short-tailed Albatross (*Phoebastria albatrus*). Laysan (*P. immutabilis*) and Black-footed Albatrosses (*P. nigripes*) were used as the outgroup. Bootstrap values over 50% obtained from 1000 resamplings are shown. Clade B was supported by a 47% bootstrap value. HS, HC, and TC represent unringed subadults from Hatsunezaki, chicks from Hatsunezaki, and chicks from Tsubamezaki, respectively. AB254197–AB254200, AB254205–AB254206, AB254211, AB254217–AB254222, AB254226–AB254228, AB254230, AB254232, AB254234–AB254235, LC534780–LC534781: sequences from birds in Torishima; *AB254238: sequence from birds in the Senkaku Islands; LC066675–LC066677, LC534782: sequences from unringed birds at Hatsunezaki.

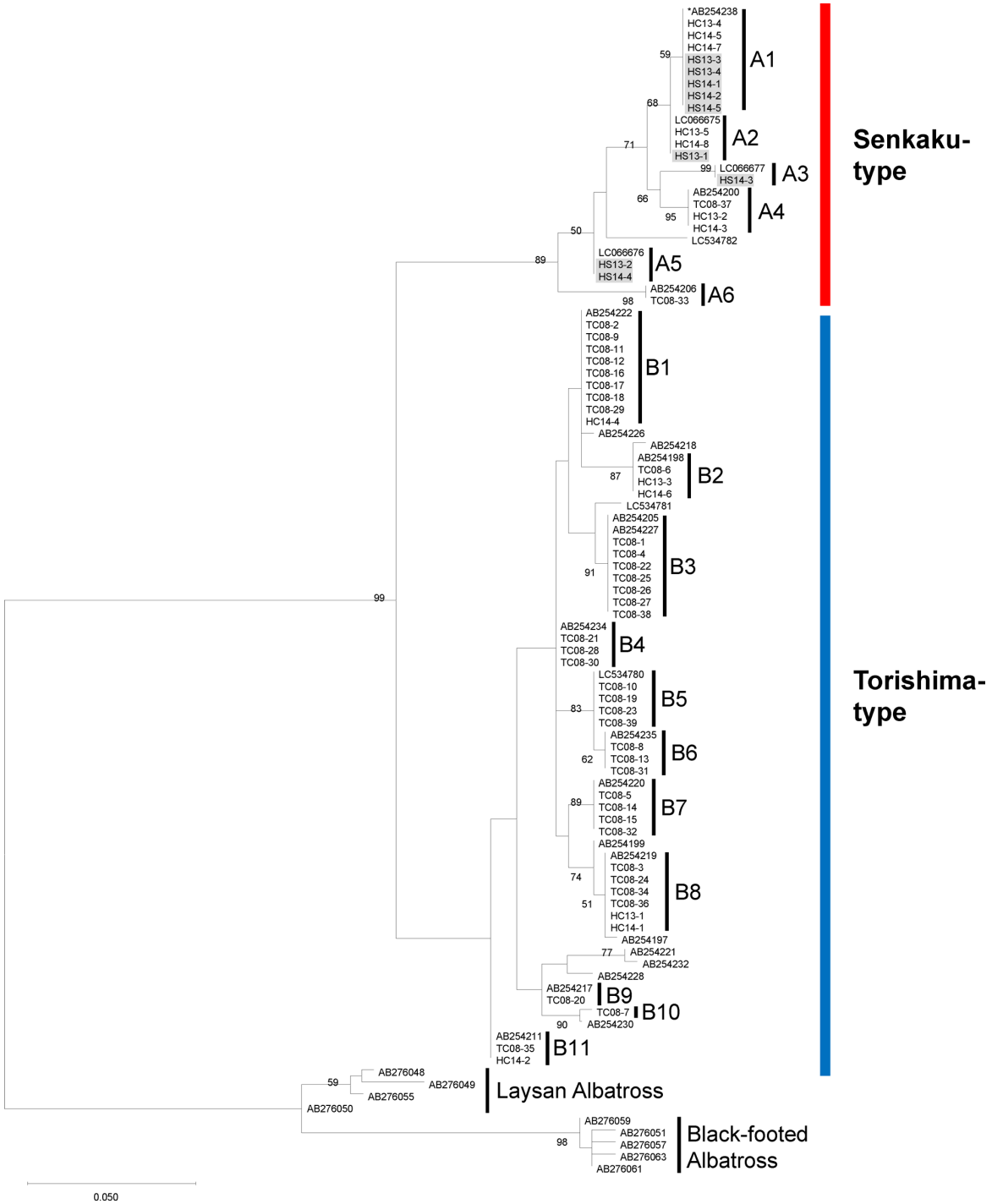
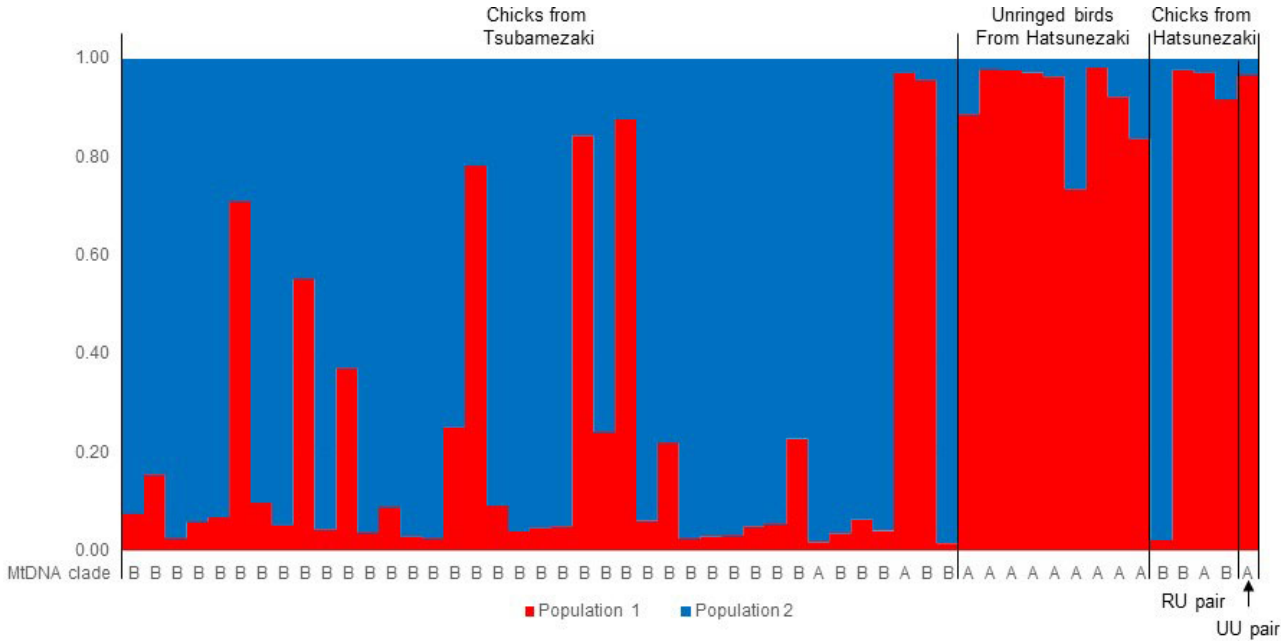


Fig. 3. Bayesian clustering of 10 microsatellite loci genotypes of individuals performed in STRUCTURE with $K = 2$. Each individual is represented by a vertical line with the proportion of alleles from different populations. The mitochondrial DNA clade of each individual is also shown. RU: ringed–unringed parent pair; UU: unringed–unringed parent pair.



and the estimated proportion of alleles from each population was similar in siblings. The other bird had mixed ancestry, differing from its sibling by ~17% in the proportion of alleles derived from each population. A sibling from the UU pair had the clade B CR2 sequence but a high proportion of alleles from population 1.

DISCUSSION

MtDNA and nuclear DNA differences between the two cryptic species

The ML tree constructed from mtDNA CR2 sequences revealed two major distinct clades in the Short-tailed Albatross, as shown in previous studies (Kuro-o et al. 2010, Eda et al. 2011, 2012, 2020). All nine unringed birds had sequences belonging to clade A, whereas 37 of 39 chicks from Tsubamezaki had sequences belonging to clade B. These findings reconfirmed that the birds from the Senkaku Islands and Torishima have formed different genetic populations for a long time. Assuming an evolutionary rate of 1.58% myr^{-1} for the third codon of the cytochrome *b* region sequence (Nunn et al. 1996), the genetic divergence between the two lineages occurred ~638,000 years ago (Eda 2018).

In the microsatellite DNA analysis, the indicators of genetic diversity were larger in chicks from Tsubamezaki than in unringed birds, suggesting higher genetic diversity in the Torishima population than in the Senkaku Islands population. A difference in genetic diversity between the two populations has also been observed in maternal mtDNA analysis (Eda and Higuchi 2012). Over all loci, F_{IS} values were significantly positive in chicks from Tsubamezaki and Hatsunezaki, suggesting the Wahlund effect, a reduction in heterozygosity in a population caused by subpopulation structure. The observed deviation from linkage equilibrium in two pairs of loci in chicks from Tsubamezaki also suggests the existence of two genetic populations in the group.

Based on the optimal populations ($K = 2$) inferred using STRUCTURE analysis, most unringed birds captured at the Hatsunezaki colony (~90%) and a large proportion of chicks hatched at the Tsubamezaki colony (~60%) displayed high proportions of alleles from populations 1 and 2, respectively. These findings suggest that the birds from the Senkaku Islands and Torishima have largely formed different genetic populations and that the nuclear DNA composition supports the existence of cryptic species of the Short-tailed Albatross, in addition to the previous morphological, ecological, and maternally inherited mtDNA evidence (Eda and Higuchi 2012, Eda et al. 2016, 2020).

Hybridization of the two cryptic species

In general, Senkaku-type albatrosses had CR2 sequences belonging to clade A and a high proportion of alleles from population 1, whereas Torishima-type albatrosses had CR2 sequences belonging to clade B and a high proportion of alleles from population 2. There were no observations of pairing of unringed subadult plumage birds (F.S., *personal observation*), suggesting that there have not been any recent immigrants from the Senkaku Islands to Tsubamezaki. However, clade A CR2 sequences were observed in ~5% and ~7% of chicks hatched on Tsubamezaki in this study and a previous study (Kuro-o et al. 2010), respectively. Furthermore, in the STRUCTURE analysis, ~30% of chicks that hatched at Tsubamezaki did not have a high proportion of alleles from either population, suggesting that these individuals were affected by genetic hybridization.

Male albatrosses generally arrive at the breeding site earlier than females in the breeding season (Huyvaert et al. 2006, Jones and

Ryan 2014), and fertilization after copulation can occur for only a few days from the arrival of the female at the breeding site (Astheimer et al. 1985). The breeding season starts ~2 weeks earlier on Senkaku Islands than in Torishima (Kuroiwa 1900, Miyajima 1900, Hasegawa 2006). It could be expected to work as a post-mating and pre-zygotic isolation mechanism between the two species, especially for a pair of Torishima-type male and Senkaku-type female. Actually, a probable hybrid pair mated in an artificially re-established colony on Mukojima Island, Bonin Islands, where 70 chicks were translocated from Torishima and hand-reared during 2008 and 2012 (Deguchi et al. 2017). DNA analyses revealed that the ringed hand-reared male and unringed female had Torishima- and Senkaku-type CR2 sequences, respectively (Deguchi et al. 2017). They mated in 2012, but the male arrived after the female during the first two years and failed to breed for the first three years, longer than other pairs, likely because of infertile eggs (Deguchi et al. 2017).

At Tsubamezaki, however, one chick had a high proportion of alleles from population 2 and clade A CR2 sequences, while three chicks had a high proportion of alleles from population 1 and clade B CR2 sequences. This suggests that backcrossing has occurred in Tsubamezaki, and that pairs of Senkaku-type female and Torishima-type male, as well as Senkaku-type male and Torishima-type female, yield viable F1 hybrids. The probable hybrid pair on Mukojima also eventually succeeded in the fledging of chicks in their fourth breeding season (Deguchi et al. 2017). Therefore, the post-mating and pre-zygotic isolation mechanism was not completely established between the two cryptic species of the Short-tailed Albatross. Viable F1 hybrids have been reported in interspecies pairs in Diomedeidae, including pairs of Black-footed × Laysan albatrosses (Rohwer et al. 2014), Campbell (*Thalassarche impavida*) × Black-browed albatrosses (*T. melanophris*; Moore et al. 2001), and Northern Royal (*Diomedea sanfordi*) × Southern Royal albatrosses (*D. epomophora*; Robertson 1993). Hybridization and gene flow have also been reported between seemingly distinct seabird taxa (Brown et al. 2010, Taylor et al. 2012a, 2012b, Brown et al. 2015, Zidat et al. 2017, Jones et al. 2020).

To date, there is no evidence of successful immigration from Torishima to the Senkaku Islands, given that no ringed albatrosses were observed in field surveys in the Senkaku Islands during 2001 and 2002 (Hasegawa 2015). However, sibling chicks of a UU pair, the parents of which were suggested to have been born in Senkaku Islands, in Hatsunezaki, had a CR2 sequence belonging to clade B and a high proportion of alleles from population 1 in the additional STRUCTURE analysis. In addition, one of the unringed birds did not have a high proportion of alleles from either population. These findings suggest that at least one Torishima-type female albatross successfully immigrated to the Senkaku Islands, and that genetic hybridization and backcrossing also occurred in the Senkaku Islands.

Hybridization of the two cryptic species was suspected in Hatsunezaki, because eight of 117 nests were occupied by ringed-unringed pairs in a previous study (Eda et al. 2016). However, three and one of the four ringed-unringed pair chicks displayed a high proportion of alleles from populations 1 and 2, respectively. The trend was similar in the additional STRUCTURE analysis, and no apparent proof of extra-pair mating was found in any of the chicks. The unringed parent of the latter chick was likely to be a Torishima-hatched individual that lost its ring(s) or escaped ringing and had a high proportion of alleles from population 2. This unringed bird paired with a ringed Torishima-type bird from Torishima. The ringed parent of the former three chicks, including the mate of Deko-chan, are considered to be Torishima-hatched individuals with a high proportion of alleles from population 1, which paired with unringed Senkaku-type birds from the Senkaku Islands. Their ancestors seemed to have immigrated to Torishima from Senkaku Islands (or other region[s] where population 1 birds inhabited) and mainly mated with birds with a high proportion of population 1 alleles. This suggests that two of the three pedigrees, including the pedigree of the mate of Deko-chan, historically mated with a female with clade B CR2 and some extent of alleles from population 2, but the alleles from population 2 were lost by subsequent mating with birds with a high proportion of alleles from population 1. The reason why Deko-chan did not pair with a live albatross but behaved as though it was paired with a particular decoy for eight years or until the time of decoy removal (Sato 1999, Eda et al. 2011) could be explained by the scarcity of Senkaku-type females in Hatsunezaki. Because all the four studied ringed-unringed pairs were suggested to mate in an assortative manner—although it is possible that the genetic and apparent fathers of some ringed-unringed pair chicks were different and the genetic fathers were of the same type as the mothers—all cases were not regarded as hybridization of the cryptic species. Similarly, the other four ringed-unringed pairs observed in Eda et al. (2016) might also mate assortatively, and therefore, hybridization of the cryptic species might not occur in Hatsunezaki. It is obvious that the pre-mating isolation between the two cryptic species in Hatsunezaki is much stronger than that suggested by Eda et al. (2016).

Evolutionary consequence of secondary contact between the two cryptic species

Following a secondary contact of allopatric sister species, several evolutionary consequences are expected: reinforcement of reproductive isolation, hybrid speciation, de-speciation, introgressive hybridization, or formation of a stable hybrid zone (Seehausen 2004, Jacobsen and Omland 2011, Hasegawa 2012, Brown et al. 2015, Grant and Grant 2019). The observed patterns of backcrossing in Torishima and the Senkaku Islands and assortative mating of each species in Torishima suggested that hybrid individuals were not preferred in Torishima or the Senkaku Islands. Therefore, hybrid speciation and de-speciation seems unlikely, at least in the current situation.

In Tsubamezaki, some chicks had a medium proportion of alleles from populations 1 and 2, suggesting current hybridization. In contrast, all the chicks obtained from four ringed-unringed pairs at Hatsunezaki displayed a high proportion of alleles from either population 1 or 2, not suggesting ongoing hybridization. Breeding of unringed subadult plumage birds was observed in Hatsunezaki but not in Tsubamezaki (F.S., *personal observation*). In contrast, in Tsubamezaki, the breeding of many unringed elder albatrosses, which likely hatched before 1979 and are of unknown origin, has been observed (Hasegawa 2015). Diomedeidae albatrosses, including the Short-tailed Albatross, are monogamous and maintain a pair-bond until one member dies (Tickell 2000, Hasegawa 2015). Therefore, it could be possible that pairing of

individuals from different cryptic species may have occurred in the past but not recently in Torishima. If this is the case, this trend could be explained by the reinforcement of reproductive isolation. Further microsatellite DNA studies of chicks from Hatsunezaki are required to confirm whether reinforcement of reproductive isolation is achieved. The reinforcement of reproductive isolation over time was suggested in hybrid zones of the Lazuli Bunting (*Passerina amoena*) and Indigo Bunting (*P. cyanea*; Carling and Zuckerberg 2011), the Pied Flycatcher (*Ficedula hypoleuca*) and Collared Flycatcher (*F. albicollis*; Sætre and Sæther 2010), the Great Black-backed Gull (*Larus marinus*) and American Herring Gull (*L. argentatus smithsonianus*; Sternkopf et al. 2010), as well as the Willow Flycatcher (*Empidonax traillii*) and Alder Flycatcher (*E. alnorum*; Bemmels et al. 2021).

Alternatively, the current level of or nearly complete pre-mating isolation between the two species was established, but the scarcity of Senkaku-type birds in Torishima facilitated the past hybridization. In hybrid zones, especially for female birds, it may be better to produce at least some viable F1 rather than remain unpaired and abandon reproduction (Baker 1996, Veen et al. 2001). The number of Short-tailed Albatross drastically declined as a result of hunting for feather collection during the late 19th and early 20th century (Tickell 2000, Hasegawa 2003, U.S. Fish and Wildlife Service 2008), and their extinction was declared (Austin 1949). In the middle of the 20th century, the populations of Short-tailed Albatross in Torishima and the Senkaku Islands were estimated to be ~60 and ~15, respectively (Eda and Higuchi 2012, Hasegawa 2015). If Senkaku-type males (or females) were absent or already paired in Torishima, a Senkaku-type female (or male) may have chosen a Torishima-type male (or female) as "making the best of a bad job" (Randler 2008). When the probable hybrid pair on Mukojima mated in 2012, only 10 albatrosses were observed on Mukojima (Deguchi et al. 2017). The scarcity of Senkaku-type males and Torishima-type females on Mukojima may have facilitated this hybrid mating. Microsatellite DNA analysis of chicks from the ringed-unringed pair in Mukojima is required to ascertain whether they are hybrid F1s of the two cryptic species.

To predict the evolutionary consequence of a secondary contact between the two cryptic species of Short-tailed Albatross, it is essential to estimate the strength of post-zygotic isolation. Further behavioral analyses combined with molecular analyses are required to examine whether reproductive success differs between hybrid and pure line individuals. Ecological surveys in the Senkaku Islands, which have not been conducted since 2002, are also required to establish appropriate conservation policies for these species.

Author Contributions:

M.E. formulated the questions; S.K., M.K., Y.W., and F.S. conducted field research; H.I. and M.E. conducted DNA analysis; M.E. and H.I. wrote the paper; and F.S. supervised research.

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Appendix 1. The result of mtDNA analysis and STRUCTURE analysis for each sample. UU, RU, and UN indicate nests of unringed–unringed, ringed–unringed, and unringed–ring status unknown pair, respectively. *: Nest of "Deko-chan," which was the first immigrant from Senkaku Islands to Hatsunezaki and made a nest close to decoy No. 22. Information for the sex and mtDNA sequence of unringed subadults is referred from Eda et al. (2020).

Please click here to download file 'appendix1.xlsx'.

Appendix 2. Genetic diversity (A: number of alleles per locus, AR: allelic richness, NE: number of effective alleles, HE: expected heterozygosity, HO: observed heterozygosity, FIS: inbreeding coefficient) of the Short-tailed Albatross based on 10 microsatellite loci.

Please click here to download file 'appendix2.xlsx'.

Appendix 3. Linkage disequilibrium test results

Please click here to download file 'appendix3.xlsx'.

Appendix 4. Examination of allele sharing between the parent-chick pairs from Hatsunezaki

Please click here to download file 'appendix4.xlsx'.