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Annual fluctuation in Pacific bluefin tuna (*Thunnus orientalis*) larval catch from 2007 to 2010 in waters surrounding the Ryukyu Archipelago, Japan

Nobuaki SUZUKI [*1], Toshiyuki TANABE [*2], Kenji NOHARA [*3], Wataru DOI [*3], Hiroshi ASHIDA [*1], Takahiko KAMEDA [*2], and Yoshimasa AONUMA [*2]

Abstract : As the first step towards developing a better understand of the natural reproduction of Pacific bluefin tuna (PBF), research surveys were conducted in the waters surrounding the Ryukyu Archipelago in spring from 2007 to 2010. A total of 11 patches and 1,853 PBF larvae were detected using multiplex PCR genotyping specific for PBF. Marked collections of PBF larvae were taken from sites located between 24° and 26° N along a 126° E line. The majority of patches was found in slightly stagnated current regions along the Kuroshio Current or on the edge of mesoscale eddies. Based on patch distribution and oceanographic conditions, factors affecting larval catch fluctuation and a possible scenario regarding the advection process of PBF larvae were discussed.

Key words : annual fluctuation, multiplex PCR genotyping, oceanographic conditions, Pacific bluefin tuna larvae, patch distribution

Introduction

Pacific bluefin tuna (PBF) have been known to spawn in the waters around the Ryukyu Archipelago during spring and in the Sea of Japan during early summer (Yabe *et al*., 1966; Ueyanagi, 1969; Okiyama, 1974; Kitagawa *et al*., 1995; Tanaka *et al*., 2007). Previous studies have reported annual fluctuations of up to six-times total abundance in year-class strength of PBF recruitment (Yamada *et al*., 2006). Increased survival of juveniles has been considered a factor leading to dominant year classes, which have markedly contributed to PBF stock abundance, and has likely depended on larger body sizes and higher growth rates during the larval period (Tanaka *et al*., 2006). Recent radio-buoy tracking of single larval schools (patches) along with continuous sampling of PBF larvae has elucidated the fine- (100s of m to km) to mid-scale (~15 to 30 km range) structure of patches as well as the spatial distribution of larvae entrained in mesoscale eddies (100 to 500 km in diameter). This has allowed the relationship between distribution of larvae and environmental conditions, such as the role of the Kuroshio Current and mesoscale eddies, in the process of PBF recruitment to be examined (Satoh, 2010). On the other hand, reproductive ecology associated with spawning activity and abundance of wild PBF seed surrounding spawning areas remains poorly understood. When the difficulty associated with the sampling of juveniles less than 10 cm in fork length was considered, it was determined that evaluation of larval abundances may be a practical approach to the examination of annual fluctuations in wild PBF reproduction.

Research surveys in the waters surrounding the Ryukyu Archipelago have been conducted by the authors of the present study since 2007 in order to

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*1 National Research Institute of Far Seas Fisheries, FRA. 5-7-1, Orido, Shimizu, Shizuoka 424-8633, Japan
*2 Research Center for Subtropical Fisheries, Seikai National Fisheries Research Institute, Fisheries Research Agency (FRA). 148-446, Fukai-Ohta, Ishigaki, Okinawa 907-0451, Japan
*3 School of Marine Science and Technology, Tokai University. 3-20-1, Orido, Shimizu, Shizuoka 424-8610, Japan

examine natural reproduction in PBF. The objectives of the present study were (1) to develop a rapid and precise PBF identification procedure using DNA markers, (2) to determine both the larval distributions of PBF and the annual catch fluctuations in the waters surrounding the Ryukyu Archipelago, (3) to contrast larval PBF distributions with oceanographic conditions in order to consider possible factors that affected larval catch fluctuation, and (4) to discuss possible scenarios regarding wild PBF reproduction throughout the advection process of PBF larvae.

Materials and Methods

Larval sampling

Larval samples were collected through 10 minute surface tows using a 2 m diameter ring net with 335 μm mesh aboard RV "Shunyo-Maru" of the National Research Institute of Far Seas Fisheries from 2007 to 2010. Sampling was undertaken at 22, 21, 22 and 73 sites during the years 2007, 2008, 2009 and 2010, respectively. Sampling sites were originally designed using a grid; however, they were occasionally altered based on conditions at sea during actual sampling. Nets were towed at a speed of approximately 1.5 knots at night (at least one hour after sunset). Collected larvae were sorted on board and immediately preserved in 99.5% ethanol prior to a more comprehensive sorting once they had been transferred to the laboratory. Morphological observations were carried out under a microscope, and the total length of each larva was measured to the nearest 0.1 mm using digital calipers.

Oceanographic observations and data analysis

At each sampling site, sea surface temperature and temperature/salinity vertical profiles were recorded by direct measurement (0 m depth) and the use of a CTD, respectively. Sea surface height (SSH) and geostrophic current data were obtained from FRA-ROMS (http://fm.dc.affrc.go.jp/fra-roms/index.html), which was managed by the Fisheries Research Agency, Japan. Data from larval sampling, oceanographic observations and FRA-ROMS were synthesized and graphically illustrated using Generic Mapping Tools (GMT) ver. 4.5.7 (Wessel and Smith, 1998).

DNA extraction, multiplex PCR and sequencing for PBF identification

Tuna-like larvae were sorted and examined using DNA analyses in order to confirm specific identity. Crude DNA was extracted from an eye ball or from muscle tissue taken from the posterior part of larval bodies in order to prevent the extraction of contaminant DNA from the digestive tract using a Gentra Puregene Tissue Kit (QIAGEN). Identification of PBF larvae was achieved through a multiplex PCR genotyping method targeting the ATCO region of mtDNA, which have been reported to be effective in the identification of tuna species (Chow and Inoue, 1993). The tuna-universal primer pair developed by Chow and Inoue (1993) (L8562: 5'-CTT CGA CCA ATT TAT GAG CCC-3' and H9432: 5'-GCC ATA TCG TAG CCC TTT TTG-3') was used in order to amplify the target DNA fragment, which was approximately 870 base pairs (bp) in length. Two forward primers developed in the present study were also used simultaneously in a PCR reaction; L8754PNAL (5'-AGC CGT TCT ATT AAC CTC CT-3') possessed a nucleotide sequence consistent with both PBF and albacore (ALB: *T. alalunga*) and was expected to amplify ca. 680 bp of the targeted DNA fragment, while L8963PNB (5'-TTC CAG TAC TAA TTG TCA TT-3') was identical to the nucleotide sequence of PBF and was expected to amplify ca. 470 bp of the targeted DNA fragment.

PCR amplification was carried out within a 10 μl reaction mixture containing 2 mM $MgCl_2$, 0.2 mM of each dNTP, 0.17 μM of each forward primer, 0.5 μM of reverse primer, 0.25 units of Taq polymerase (TaKaRa) or AmpliTaq (Applied Biosystems), and template DNA. Cocktails were preheated at 94 ℃ for 2 min, followed by 30 cycles of amplification (94 ℃ for 30 s, 52 ℃ for 30 s and 72 ℃ for 1 min) with a final extension at 72 ℃ for 5 min. After thermal cycling, 3 μl of reaction mixtures were electrophoresed through 1.5% agarose gel and PCR products were visualized using ethidium bromide staining in order to determine ATCO genotypes.

When multiplex genotyping profiles were uncertain due to unexpected electrophoretic patterns, PCR products were subjected to direct

nucleotide sequencing. Amplified products were purified using a PCR Product Pre-Sequencing Kit (USB) and nucleotide sequences were generated on an automated sequencer (ABI Prism 310) using a BigDye Ready Reaction Kit (Applied Biosystems) and a standard cycle sequencing protocol with the H9432 reverse primer. All sequences obtained were identified and collated with DNA databases through BLAST homological search. Supplementally, partial sequences of mtDNA control regions were also determined and subjected to BLAST after PCR amplification using L15924 (5'-AGC TCA GCG CCA GAG CGC CGG TCT TGT AAA-3' : Kocher *et al*., 1993) and H16498 (5'-CCT GAA GTA GGA ACC AGA TG-3' : Meyer et al., 1990).

Results

Efficiency of multiplex PCR genotyping

In order to confirm the efficiency of the three forward and one reverse primer used, four adult *Thunnus* spp. specimens preserved in the National Research Institute of Far Seas Fisheries were examined. Three- and two-banding patterns were observed in PBF and ALB, respectively, which indicated that amplified DNA fragments of expected size were present, while a single 870 bp fragment was observed in the other species examined (Fig. 1A). In order to examine intraspecific variation, 48 adult PBF specimens were analyzed. With the exception of one specimen that showed no PCR amplification, all specimens examined were correctly identified based on their three-banded genotypic profile. These results indicated that no intraspecific variation was present (Fig. 1B).

Identification of wild-caught larvae

Multiplex PCR genotyping was carried out using wild-caught tuna-like larvae subsequent to morphological identification based primarily on melanophore distribution on the body surface. Although a few larvae possessed unexpected

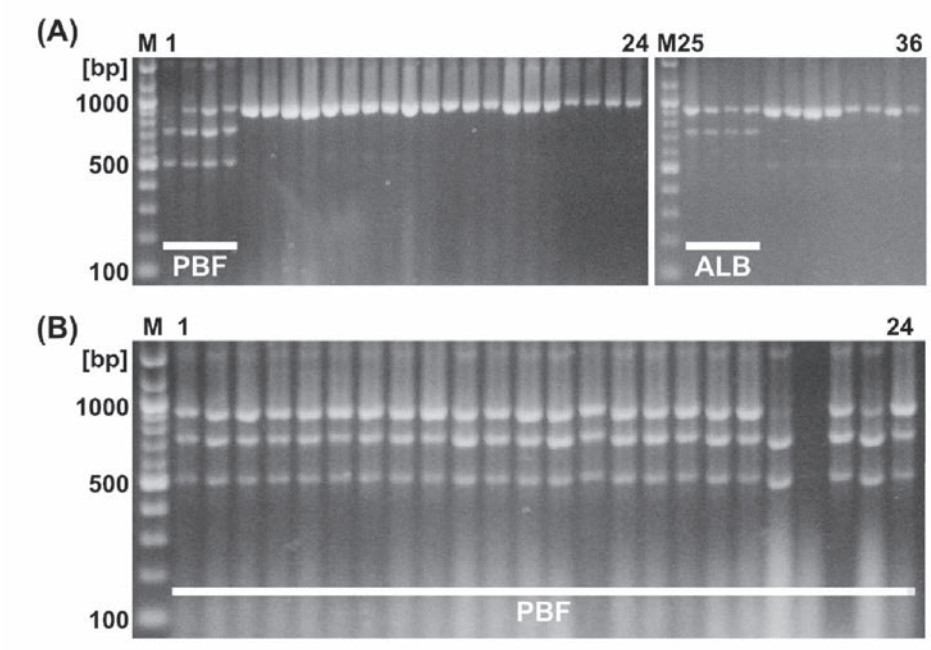

Fig. 1. Electrophoretic images of multiplex PCR products for *Thunnus* species. (A) Comparison among eight species including two known mitochondrial types of bigeye (*T. obesus*) identified by Chow et al. (2000). Lanes 1-4: Pacific bluefin, 5-8: Atlantic bluefin, 9-12: southern bluefin, 13-16: α-type bigeye, 17-20: β-type bigeye, 21-24: yellowfin, 25-28: albacore, 29-32: long tail, and 33-36: blackfin tunas. (B) Determination of intraspecific variation within Pacific bluefin tuna. In both images, lanes marked "M" include molecular size markers with sizes given along the left margin.

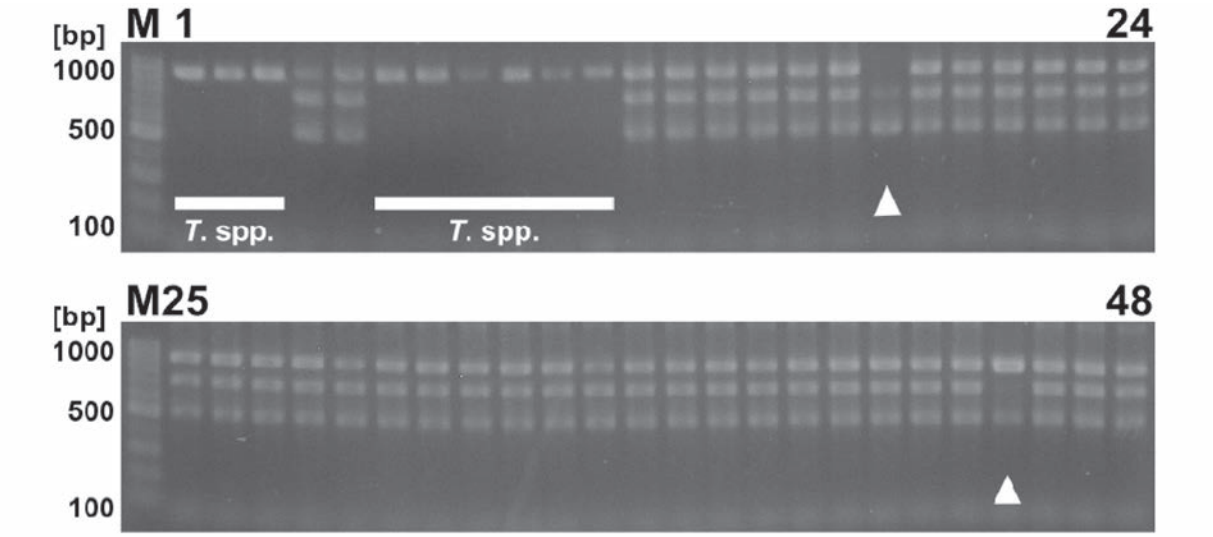

Fig. 2. Electrophoretic images of multiplex PCR products of 48 wild-caught tuna-like larvae specimens. Lanes 1-3 and 6-11, which indicate a single band, represent *Thunnus* spp. excluding Pacific bluefin. Triangles indicate variant genotypes of Pacific bluefin. Lanes marked "M" include molecular size markers with sizes given along the left margin.

electrophoretic profiles, their identities were confirmed through direct sequencing for mtDNA ATCO and control regions (Fig. 2). A total of 1,853 PBF larvae were identified based on four years worth of surveys along with 11 patches, which were defined as groups of more than ten larvae collected from a single sampling site (Table 1). A large amount of PBF larvae were collected from nine sites (St1017-1~St1018-5) on the 16th and 17th June 2010. These larvae were assigned to a single patch as they were sampled continuously using radio-buoy tracking. The total catch of PBF larvae associated with this patch consisted of 839 individuals, an average of 93.2±84.4 (mean ± SD) individuals per net.

Distribution of larval patches

Five patches were observed during both 2007 and 2009, one patch was observed during 2010, and no patches were observed during 2008. Patches identified in the present study were concentrated within an area located between Okinawa and Miyako islands, and collections of PBF larvae were obtained between 24 and 26° N along a 126° E line (Table 1, Fig. 3). Horizontal plots of patches based on geostrophic currents and SSH data both indicated that the majority of patches were located in slightly stagnated current regions along the Kuroshio Current or on the edge of mesoscale eddies (Figs. 4-7).

Discussion

In the present study, simple, rapid and precise identification of PBF larvae from among more than 1,800 tuna-like larvae was made possible using multiplex PCR genotyping. Two new primers, L8754PNAL (for PBF and ALB) and L8963PNB (for PBF), were designed to attach specific single nucleotide polymorphisms on their 3' ends, which allowed them to successfully prevent nonspecific amplification. Intraspecific variation in melanophores on larval body surfaces, which are used as a diagnostic characteristic for tuna larvae, and samples damaged by net towing can make species identification more difficult. The multiplex PCR genotyping method reported in the present study allowed the identity of PBF larvae to be confirmed using samples of varying quality and aided in the determination of wild PBF larvae abundances. This method was also capable of distinguishing PBF juveniles from those of other tuna species when morphological analysis could not. Furthermore, this multiplex PCR genotyping method could be combined with a bufferless electrophoresis system in

Table 1. Summary information regarding sampling trials and catches of Pacific bluefin (PBF) tuna larvae from 2007 to 2010. Values in bold indicate patch structures

Date	Site	Latitude (ºN)	Longitude (ºE)	SST (ºC)	No of PBF	Date	Site	Latitude (ºN)	Longitude (ºE)	SST (ºC)	No of PBF
2007/6/5	ST-2	24.00	123.50	28.4	0	2010/05/25	St1001-1	22.00	128.01	28.4	0
2007/6/6	ST-3	23.00	123.50	29.2	0		St1001-2	22.16	127.83	27.4	0
2007/6/7	ST-7	22.00	124.50	29.0	2		St1001-3	22.35	127.68	26.7	0
2007/6/8	ST-9	24.00	124.50	28.8	0	2010/05/26	St1002-1	22.99	126.99	26.7	0
2007/6/9	ST-13	25.00	126.00	25.6	**225**		St1002-2	23.16	126.82	26.8	0
2007/6/10	ST-13	25.00	126.00	27.1	2		St1002-3	23.34	126.58	26.3	0
2007/6/11	ST-14	24.00	126.00	27.1	0	2010/05/27	St1003-1	24.01	126.00	25.9	0
2007/6/12	ST-15	23.00	126.00	28.6	0		St1003-2	24.18	125.84	26.1	0
2007/6/13	ST-19	22.00	127.00	28.5	0		St1003-3	24.32	125.66	26.1	0
2007/6/14	ST-21	24.00	127.00	26.7	**23**	2010/05/28	St1004-1	25.00	125.00	26.2	0
2007/6/15	ST-23	26.00	127.00	26.2	0		St1004-2	25.17	124.83	27.4	0
2007/6/16	ST-24	27.00	127.00	28.1	**15**		St1004-3	25.35	124.67	27.6	0
2007/6/17	ST-25	29.00	128.83	26.8	**16**	2010/05/29	St1005-1	24.84	124.17	27.1	8
2007/6/18	ST-27	27.00	128.83	25.0	0		St1005-2	25.01	124.00	27.3	0
2007/6/19	ST-29	25.00	128.83	28.0	0		St1005-3	25.21	123.84	27.3	0
2007/6/20	ST-38	25.00	130.00	27.7	9	2010/05/30	St1006-1	25.33	125.67	26.9	0
2007/6/21	ST-40	27.00	130.00	25.7	0		St1006-2	25.17	125.83	25.6	0
2007/6/22	ST-48	28.36	127.46	28.3	4		St1006-3	24.98	126.00	25.5	0
2007/6/23	ST-49	27.36	127.46	27.4	2	2010/05/31	St1007-1	24.00	127.02	26.1	0
2007/6/24	ST-50	27.20	126.63	28.2	2		St1007-2	23.83	127.17	26.2	0
2007/6/25	ST-51	25.50	126.50	27.6	**48**		St1007-3	23.63	127.35	26.2	0
2007/6/26	ST-52	24.50	126.50	27.2	0	2010/06/01	St1008-1	24.99	127.17	25.7	2
							St1008-2	25.16	127.00	25.6	0
2008/6/11	ST-05	21.67	125.01	29.4	0		St1008-3	25.33	126.80	26.4	0
2008/6/12	ST-04	23.00	125.00	28.1	0	2010/06/02	St1009-1	25.68	126.33	26.5	8
2008/6/13	ST-03	23.84	124.51	29.1	0		St1009-2	25.84	126.18	25.8	0
2008/6/14	ST-06	23.50	126.01	28.5	0		St1009-3	26.02	126.00	27.2	0
2008/6/15	ST-07	24.93	126.08	27.6	1	2010/06/03	St1010-1	26.00	127.00	26.5	0
2008/6/16	ST-08	25.01	125.04	27.7	2		St1010-2	26.17	126.83	26.3	1
2008/6/17	ST-24	25.49	125.96	28.1	1		St1010-3	26.35	126.66	26.1	0
2008/6/18	ST-25	25.09	125.93	28.0	2	2010/06/04	St1011-1	26.68	126.34	27.6	0
2008/6/19	ST-09	25.09	124.01	28.7	4		St1011-2	26.86	126.19	27.4	1
2008/6/20	ST-12	26.42	126.51	28.4	0		St1011-3	27.05	126.03	25.2	0
2008/6/21	ST-13	27.01	126.96	28.4	0	2010/06/05	St1012-1	27.68	127.32	27.1	0
2008/6/22	ST-14	27.68	127.26	28.4	2	2010/06/15	St1016-1	24.65	126.34	26.8	2
2008/6/23	ST-15	28.34	127.75	27.9	0		St1016-2	24.84	126.17	26.7	0
2008/6/24	ST-16	29.01	128.17	27.8	0		St1016-3	25.04	126.01	26.6	0
2008/6/25	ST-17	29.25	128.67	27.8	0	2010/06/16	St1017-1	24.95	125.99	26.7	**122***
2008/6/26	ST-26	30.33	128.92	25.4	0		St1017-2	24.87	125.99	26.8	**87***
2008/6/27	ST-27	30.33	129.85	26.9	0		St1017-3	24.91	126.05	26.8	**275***
2008/6/28	ST-28	29.84	131.35	27.5	0		St1017-3-2	24.91	125.95	26.8	**33***

Table 1. Continued

Date	Site	Latitude (ºN)	Longitude (ºE)	SST (ºC)	No of PBF	Date	Site	Latitude (ºN)	Longitude (ºE)	SST (ºC)	No of PBF
2008/6/29	ST-19	27.01	129.99	28.3	0	2010/06/17	St1018-1	25.04	126.05	26.8	**16***
2008/6/30	ST-30	27.76	126.51	28.0	0		St1018-2	25.08	126.06	26.9	**106***
2008/7/1	ST-31	27.01	125.89	28.9	0		St1018-3	25.04	126.10	26.9	**9***
							St1018-4	25.00	126.06	26.9	**40***
2009/5/31	ST-16	22.00	125.00	26.6	0		St1018-5	25.04	126.02	26.7	**151***
2009/6/1	ST-17	23.50	125.00	27.0	3	2010/06/18	St1019-1	24.51	123.50	26.5	1
2009/6/2	ST-19	26.08	125.00	26.8	7		St1019-2	24.67	123.34	26.8	5
2009/6/3	ST-20	25.25	125.00	26.3	7		St1019-3	24.84	123.16	28.2	0
2009/6/4	ST-10	24.50	125.00	27.0	9	2010/06/19	St1020-1	24.67	122.68	28.6	2
2009/6/5	ST-15	23.50	123.50	28.0	6		St1020-2	24.76	122.84	28.5	2
2009/6/6	ST-21	25.17	124.00	26.7	6		St1020-3	24.76	123.01	27.8	0
2009/6/7	ST-8	24.00	126.00	27.5	**48**	2010/06/20	St1021-1	24.67	124.34	26.9	8
2009/6/8	ST-7	25.08	126.08	26.9	**24**		St1021-2	24.83	124.17	27.1	1
2009/6/9	ST-22	23.33	126.15	27.1	1		St1021-3	25.02	124.03	26.8	2
2009/6/10	ST-23	26.15	126.00	27.2	1	2010/06/21	St1022-1	26.17	126.34	27.2	2
2009/6/11	ST-4	28.50	127.00	24.5	0		St1022-2	26.34	126.17	28.0	0
2009/6/17	ST-8a	24.00	126.00	28.1	0		St1022-3	26.54	126.05	27.7	3
2009/6/18	ST-7a	25.08	126.08	27.3	**125**	2010/06/22	St1023-1	28.33	128.17	26.2	0
2009/6/19	ST-20a	25.25	125.00	29.4	7		St1023-2	28.50	128.01	27.3	0
2009/6/20	ST-21a	25.17	124.00	29.4	5		St1023-3	28.70	127.87	27.4	0
2009/6/21	ST-23a	26.15	126.00	27.7	**324**	2010/06/23	St1024-1	28.01	127.51	27.0	2
2009/6/22	ST-3a	27.00	127.00	28.2	**18**		St1024-2	28.17	127.35	27.6	0
2009/6/23	ST-2a	25.50	127.00	27.8	7		St1024-3	28.33	127.21	28.2	0
2009/6/24	ST-10a	24.50	124.00	28.6	4	2010/06/24	St1025-1	28.18	127.33	27.6	0
2009/6/25	ST-24a	26.42	126.00	28.4	0		St1025-2	28.34	127.50	27.5	0
2009/6/26	ST-25a	28.00	127.00	27.9	2		St1025-3	28.51	127.59	27.4	0
						2010/06/25	St1026-1	28.51	127.50	27.4	0
							St1026-2	28.68	127.67	26.9	0
							St1026-3	28.85	127.76	27.3	0
						2010/06/26	St1027-1	29.18	128.18	27.6	0
							St1027-2	29.26	128.44	27.6	0
							St1027-3	29.34	128.36	27.6	0

SST: Sea surface temperature

* A total of 839 PBF larvae were assigned to a single patch as continuous sampling using radio-buoy tracking was employed.

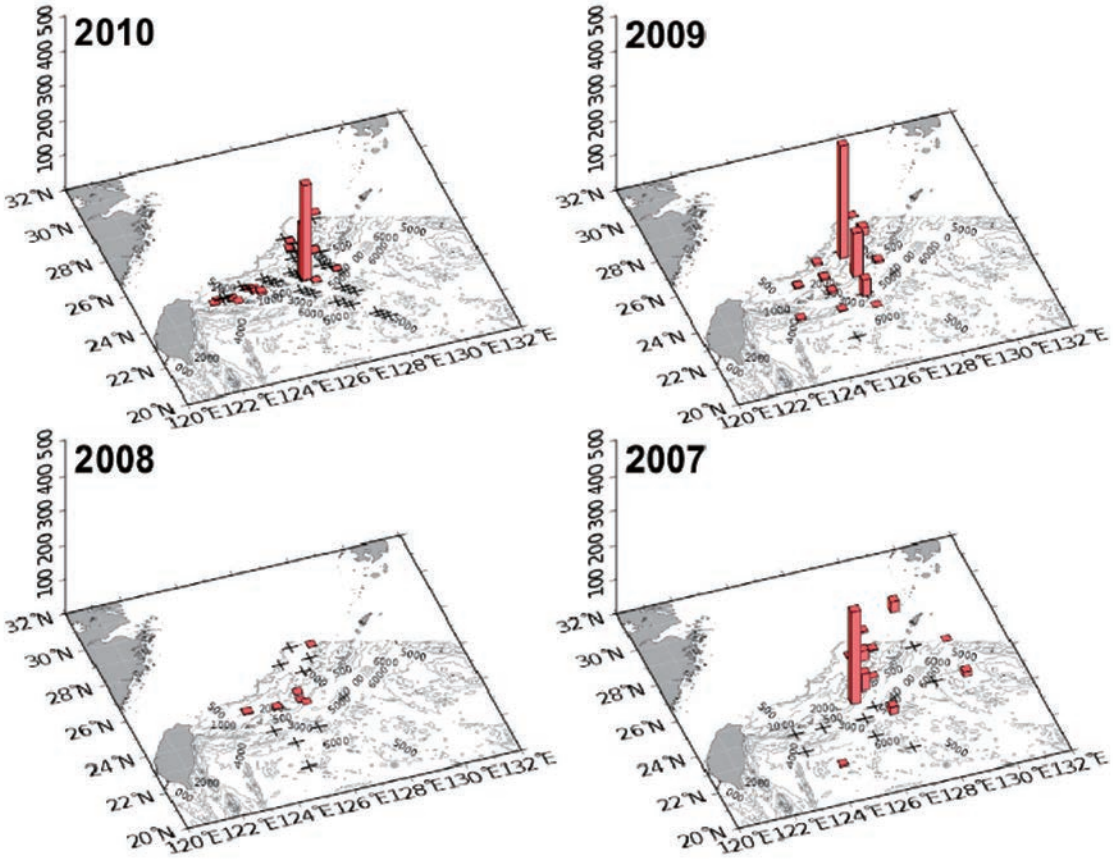

Fig. 3. Horizontal plots of annual catches of Pacific bluefin larvae from 2007 to 2010. Red bars indicate numbers of larvae and "+" indicates that no catch was obtained at a given site.

order to analyze PBF larvae and juveniles on board research vessels and could be applied to several fields of research regarding PBF ecology.

Oceanographic conditions throughout the duration of surveys conducted in 2008 and 2010 were characterized by high sea-levels in a region south of Ishigaki and Miyako islands, where a mesoscale anticyclonic (clockwise) eddy appeared to produce southward or southeastward currents between Okinawa and Miyako islands (Figs. 5 and 7). These conditions may have caused less detection of larval patches during 2008 and 2010 in this region. Patches identified during 2007 and 2009 were observed within a larger area in early and late June (Figs. 4 and 6). Patch distribution during these two years suggested that advection into the Kuroshio Current from the region south of the spawning area occurred via the waters between Okinawa and Miyako islands. Satoh (2010) suggested that the westward propagation of mesoscale eddies played an important role as a means by which larvae and early juveniles with poor swimming ability could approach the upstream Kuroshio Current. Results of the present study from 2007 and 2009 may have indicated an alternative northward advection process that resulted in the early migration of PBF larvae into the Kuroshio Current. Substantial collection of PBF larvae during 2007 and 2009, contrasted with the relatively few samples collected during 2008 and the single patch identified during 2010, might be also influenced by bottom topography and its surrounding local currents. Several patches existed along the southern edge of a shallow reef (< 100 m depth) located on the eastern region of Miyako Island where the bottom slopes dropped rapidly to a depth of 1,000 m (Fig. 8). When northward currents meet with the reef, upwelling and/or small but complicated local eddies may have been generated, producing complex conditions within the local environment. Raw current data collected using an

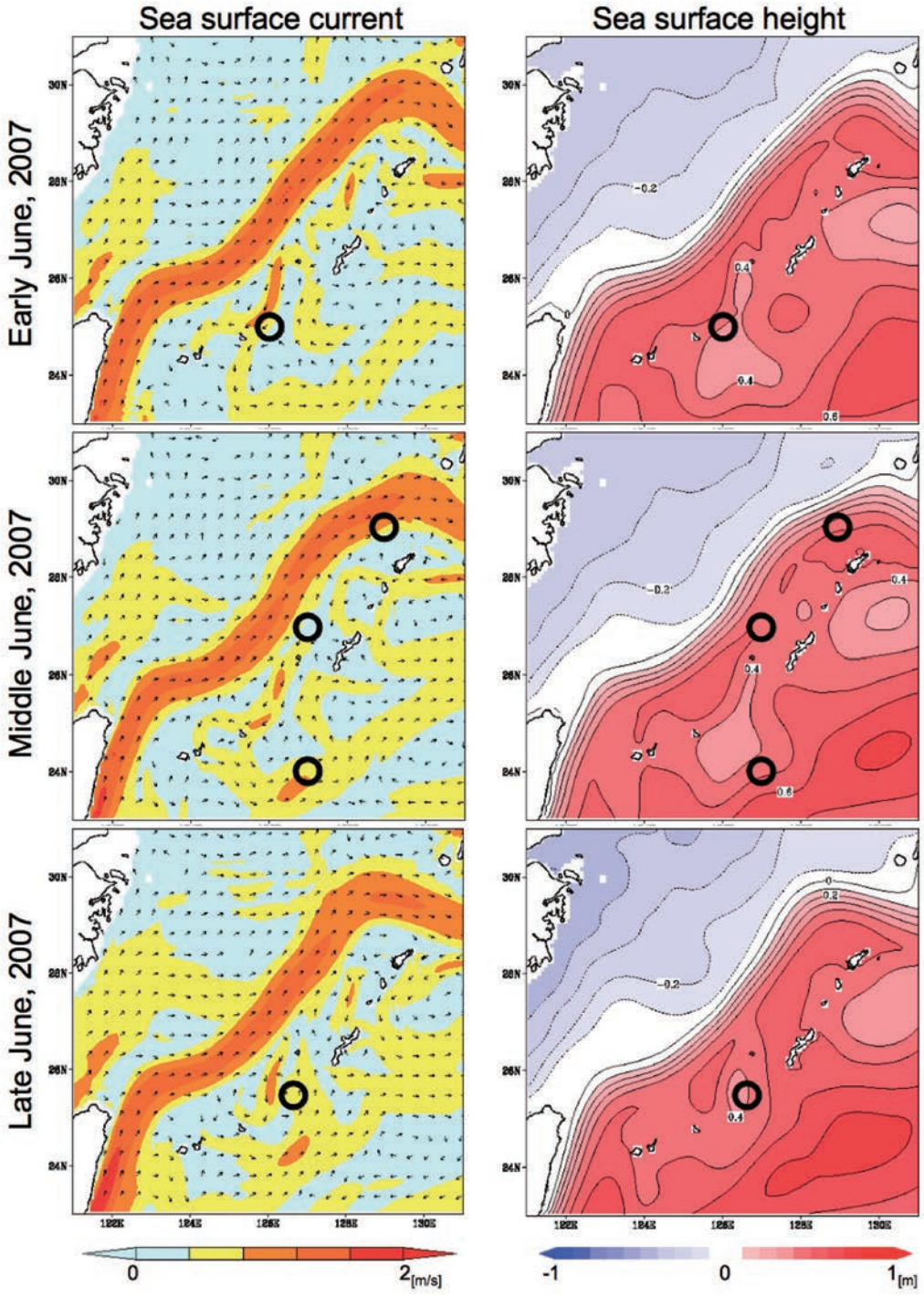

Fig. 4. Contour plots of sea surface geostrophic currents and sea surface heights with distribution of larval patches of Pacific bluefin tuna during June 2007. Open circles indicate larval patches.

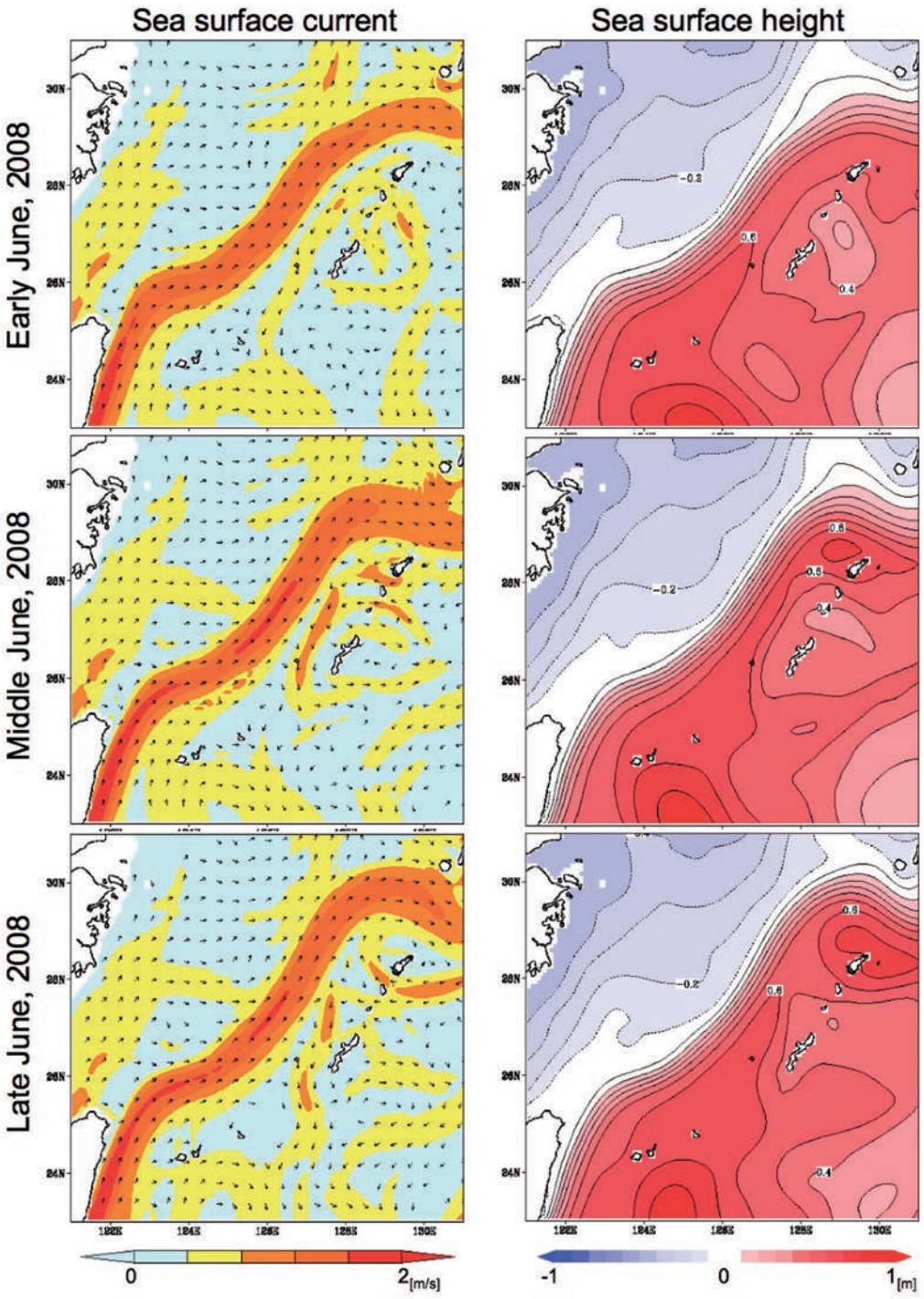

Fig. 5. Contour plots of sea surface geostrophic currents and sea surface heights during June 2008.

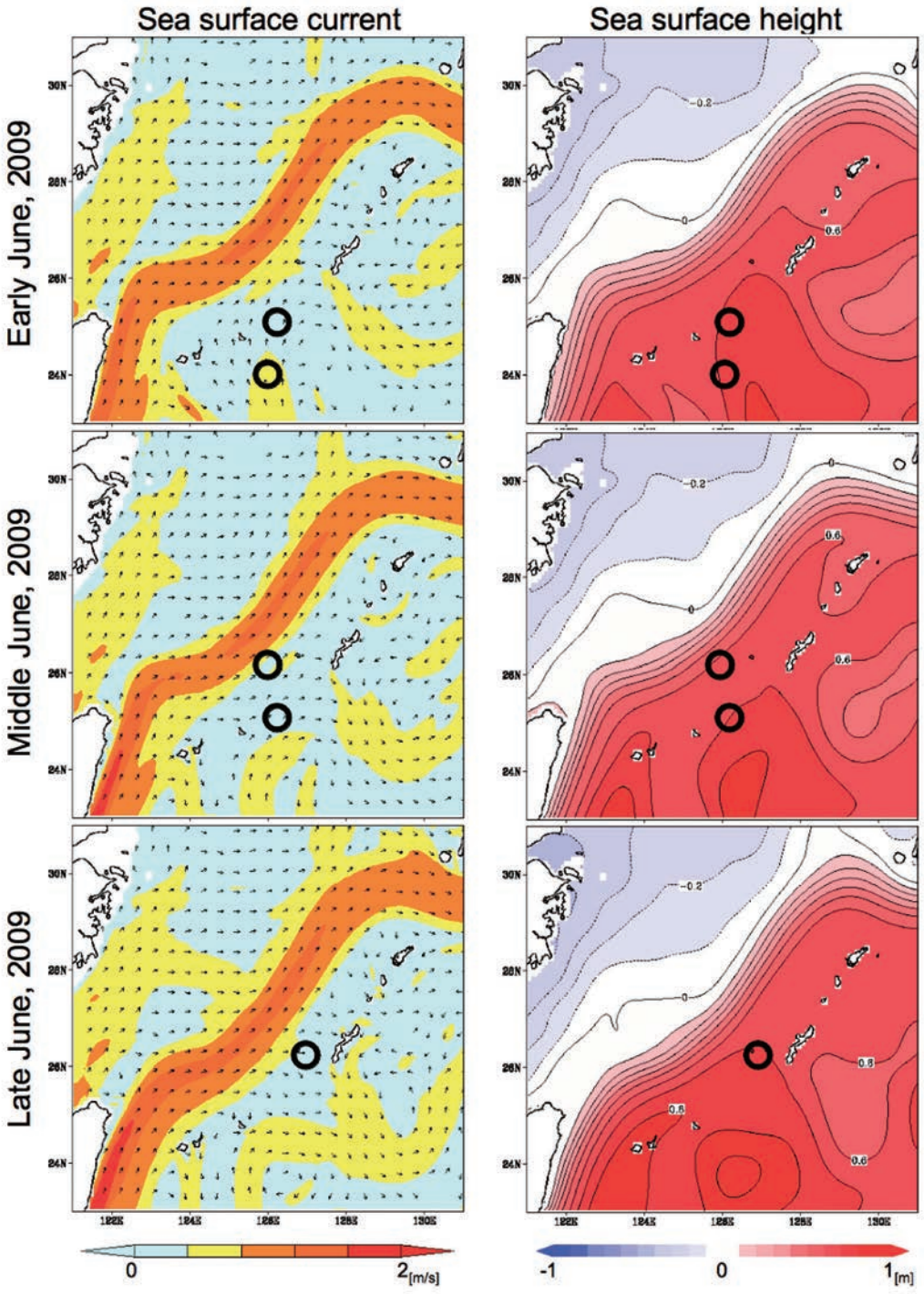

Fig. 6. Contour plots of sea surface geostrophic currents and sea surface heights with distribution of larval patches of Pacific bluefin tuna during June 2009. Open circles indicate larval patches.

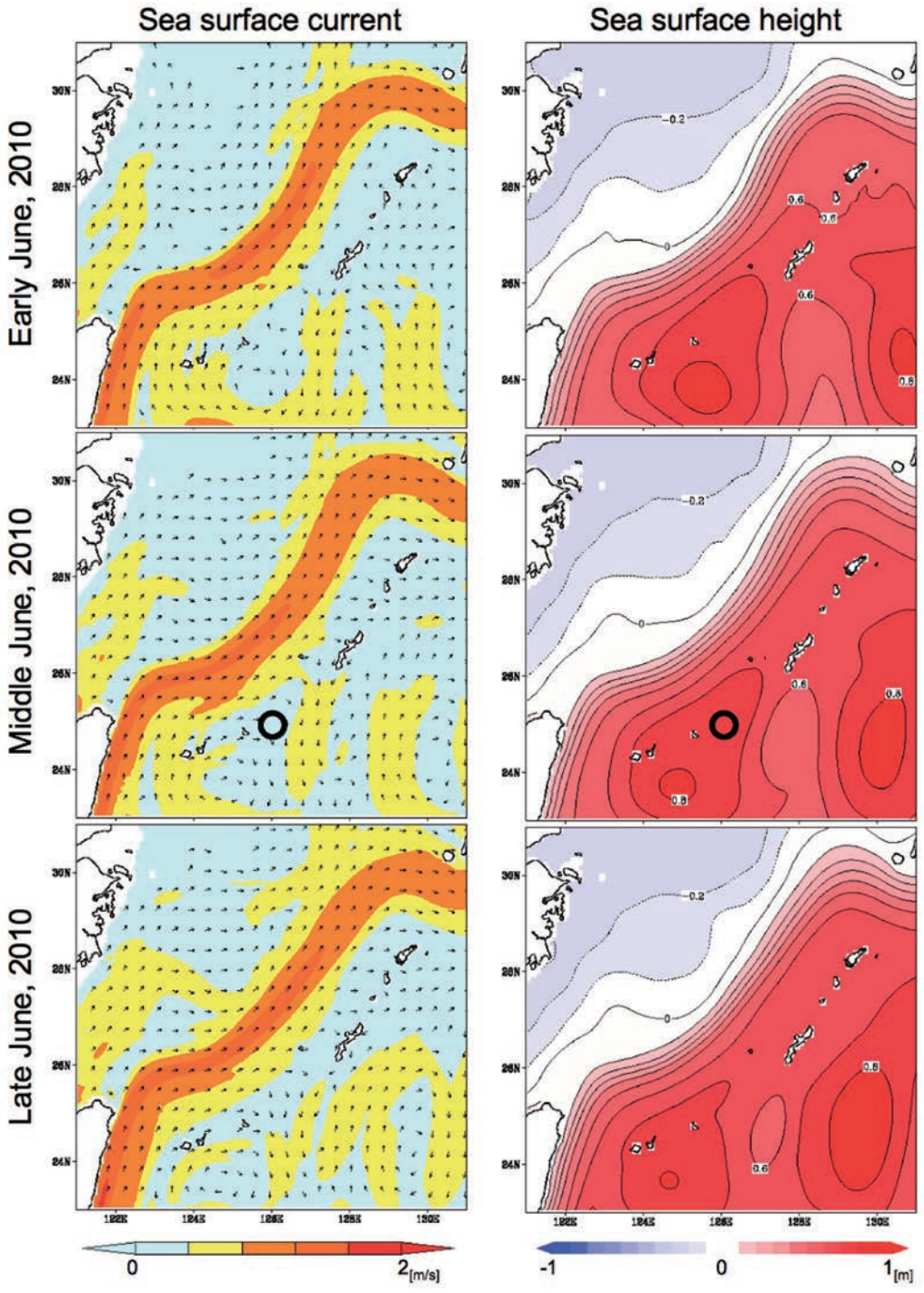

Fig. 7. Contour plots of sea surface geostrophic currents and sea surface heights with distribution of larval patches of Pacific bluefin tuna during June 2010. Open circles indicate larval patches.

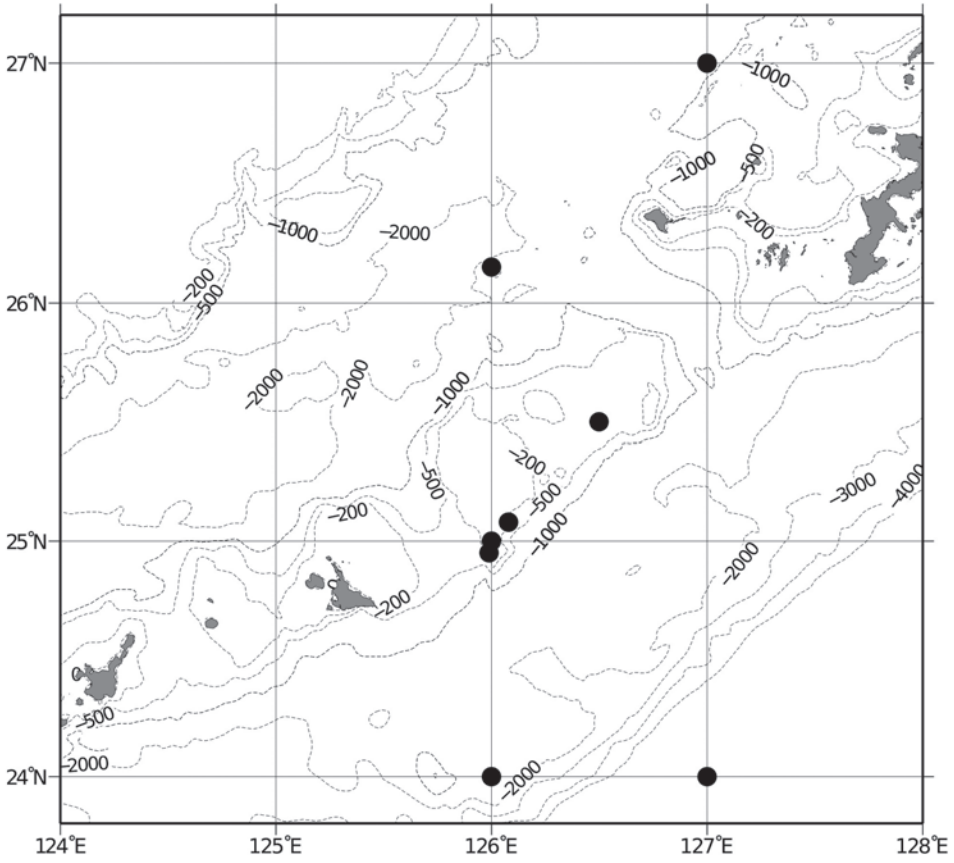

Fig. 8. Bathymetry and distribution of larval patches of Pacific bluefin tuna within the region between Miyako and Okinawa islands. Solid circles indicate larval patches.

acoustic Doppler current profiler (ADCP) demonstrated this complex structure as well as the presence of an eddy within the region (data not shown). Further study should be undertaken in order to accumulate CTD data and ADCP profiles as well as to illustrate environmental conditions that might explain the relationships between occurrence of larvae and various oceanographic factors. Furthermore, examination of larval retention by local eddies within the region examined in the present study may allow more accurate sampling methods to be designed for PBF larvae. The region examined in the present study would also serve as an ideal location for monitoring the northward advection of PBF larvae.

In the present study, horizontal distribution and annual fluctuation in PBF larval catch as well as oceanographic conditions during survey periods were determined, and a possible scenario regarding the advection process of PBF larvae was presented. Bakun (2006, 2012) summarized potential scenario of reproductive scheme of bluefin tunas and discussed the relationships between reproductive success and spawning strategy. Both in the Gulf of Mexico and in the western Mediterranean, primary but detailed analyses combining distributions of Atlantic bluefin tuna larvae with the environmental factors have been conducted (Teo *et al*., 2007; Alemany *et al*., 2012). In order to better understand reproduction in wild PBF, further study regarding PBF larvae is required following these previous studies.

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