Species identification of larval fish in Hawaiian waters using DNA barcoding

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Research Article

Keywords: molecular identification, fish larvae, DNA barcoding, diversity, Hawaiian waters

Posted Date: November 9th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-953959/v1

Abstract

Background: Correct identification of fish larvae is notably difficult, given that the morphological features at this stage of development are often poorly defined. However, obtaining such taxonomic information is of considerable importance from the perspectives of fisheries management and conservation.

Results: In this study, we collected a total of 95 specimens of larval fish from Hawaiian waters, which we sought to identify using DNA barcoding based on mitochondrial DNA cytochrome oxidase subunit I sequences. Among these, 40 and 52 samples were accordingly identified to the species and genus levels, respectively. The determined average Kimura-2-parameter distances within species, genera, families, and orders were 0.72%, 25.99%, 26.30%, and 27.50%, respectively, and the mean interspecific distance was found to be 36-fold higher than the mean intraspecific distance.

Conclusions: The results of this study provide convincing evidence that DNA barcoding can serve as an effective tool for the accurate species identification of larval fish in Hawaiian waters, and could thereby make a valuable contribution to species diversity surveys. The findings of this study can make a valuable contribution to our knowledge of the diversity and dispersal of larval fish in Hawaiian waters.

Background

The larval stage of fish is defined as the life stage prior to attaining the full complement of fin ray elements and the complete development of scales. Given that they represent a key trophic link between plankton and higher predators, the larvae of fish play an important role in the efficient functioning of marine ecosystems (Ardura et al., 2016; Heimeier et al., 2010; Mazaheri Kouhanestani et al., 2020). Moreover, larval fish are the basis for the replenishment and sustainable use of fish stocks. Consequently, a thorough understanding of the biology of the early life history of fish is essential for effective fisheries management (Mazaheri Kouhanestani et al., 2020). Specifically, the knowledge of larval ecology provides fundamental information on the reproductive biology of fish, such as the timing and sites of reproduction, migration routes, and success of population recruitment (Wibowo et al., 2017; Wibowo et al., 2018), which are important with respect to monitoring fish ecology, analyzing environmental impacts, developing management and conservation plans, establishing fishing management strategies, and contributing to the conservation of vulnerable and threatened species (Reynalte-Tataje et al., 2011; Wibowo et al., 2018).

The classification and identification of fish larvae are not only of importance with respect to taxonomic studies but also key prerequisites from the perspective fishery management. Ecological studies on the diversity and distribution of the larvae of marine fish require accurate taxonomic identification, which can, however, represent a considerable challenge (Heimeier et al., 2010; Valdez-Moreno et al., 2010). Traditionally, larval identification has been based primarily on the examination of morphological characters such as body shape, pigmentation, meristic count, and trait measurements (Azmir et al., 2017). However, a notable difficulty associated with morphological identification is that larval fish frequently bear little resemblance to the adults. Moreover, the larvae of different species often exhibit the same or duplicated characters (Ko et al., 2013; Kwun 2018; Victor et al., 2009). In particular, closely related taxa, such as congeneric and cryptic species, can be notably difficult to identify on the basis of morphology (Kwun 2018; Matarese et al., 2011). Furthermore, different levels of expertise and capabilities among larval fish taxonomists have made such experience a dependent variable in the morphological identification of larvae (Azmir et al., 2017). Consequently, at best, the larvae of most fish species can be morphologically identified with ease only to the level of phylum or class, and rarely to order and family levels. Such limitations inherent in morphology-based identification systems and the declining number of experienced taxonomists accordingly highlight the necessity of a molecular approach to the identification of larval fish up to the species level (Xing et al., 2018; Zhang and Hanner, 2011).

DNA-based techniques are useful tools that can be used to overcome the problems associated with morphological identification (Armani et al., 2015; Zeng et al., 2019). In this regard, DNA barcoding, the sequencing of an approximately 650-base pair (bp) region of the cytochrome *c* oxidase I (COI) gene, has gained widespread acceptance in the scientific literature as

an accurate, sensitive, rapid, and standard method for the identification of a diverse range of animal lineages, including fish species (Xing et al., 2018; Hebert et al., 2003; Kenchington et al., 2017; Xing et al., 2020a; Xing et al., 2020b).

The waters surrounding the Hawaiian Islands are prominent for their diverse complex physical and chemical environments. For example, physical oceanographic features, such as islands and eddies, may create favorable conditions for fish spawning, larval survival, and growth. However, little is currently known regarding the distribution, abundance, ecology, and behavior of the early stages of those species inhabiting Hawaiian waters (Paine et al., 2007).

From the perspective of promoting the sustainability of fisheries and efficient management of fish stocks, we maintain that the identification of larval fish should be implemented as one of the most basic prerequisites underpinning fisheries management. To this end, in the present study, we evaluated the utility DNA barcoding in establishing the taxonomy of larval fish collected from Hawaiian waters. We believe the findings of this study will significantly enhance our understanding of the species diversity and distribution of larval fish and the reproductive activity of fishes in Hawaiian waters, and we anticipate that our work will make a valuable contribution to documenting and conserving the biodiversity in this region.

Results

A total of 95 larvae, representing a range of different fish families and ontogenetic stages, were collected for molecular identification, among which, three samples failed to yield an amplified PCR product despite amplification using different combinations of primers and annealing temperatures. The remaining 92 samples were successfully barcoded and categorized to 12 families, 18 genera, and 20 species (GenBank accession numbers and taxonomic data are listed in Table 1), based on BLAST searches of the NCBI database. Among the 20 species, 3 (52 individuals) could not be matched to corresponding species in the database and could only be assigned to the genus at the lowest taxonomic level.

Table 1 Species composition and diversity of fish larvae collected from four sampling stations of Hawaiian waters

Family	Genus/Species	Sample ID	No.	GenBank		
				Accession numbers	Reference Sequences	Identity(%)
Hemiramphidae	*Oxyporhamphus micropterus*	B2-10/B2-17/B2-22	3	MZ028360-MZ028362	KX769054	99.34-99.56
Myctophidae	*Bolinichthys distofax*	B2-13/B3-01/B1-01	3	MZ028411-MZ028413	AP012232	99.34-99.69
	Bolinichthys sp.	B3-02/B3-03/B3-15	3	MZ028420-MZ028422	KJ967925	96.36-96.63
	Ceratoscopelus warmingii	B1-05/B1-11/ B1-14 /B1-15	4	MZ028431-MZ028434	KJ555341/KJ555342	99.54-100
	Diaphus mollis	B2-06/B4-03	2	MZ028353/MZ028354	MF041522	99.69
	Diaphus anderseni	B1-03/B1-06/ B1-07 /B1-10	4	MZ028427-MZ028430	KJ555345	99.24-99.69
	Lampadena luminosa	B2-18/B1-04	2	MZ028418/MZ028419	KJ968134/KJ555406	99.52-99.69
	Lampanyctus nobilis	B2-02/B2-09	2	MZ028347/MZ028348	MG856571	98.55
	Myctophum obtusirostre	B2-12/B3-18	2	MZ028409/MZ028410	KJ555431	99.03
Chiasmodontidae	*Kali* sp.	B1-12/B1-13	2	MZ028435/MZ028436	EU148218/MT323746	93.39-94.62
Coryphaenidae	*Coryphaena equiselis*	B3-11/B3-16	2	MZ028425/MZ028426	MH638793	99.38-99.45
Gempylidae	*Gempylus serpens*	B2-07/B2-20	2	MZ028355/MZ028356	AP012502	100
Istiophoridae	*Makaira nigricans*	B2-03/B2-21	2	MZ028349/MZ028350	KU668657	100
Mullidae	*Parupeneus* sp.	B1-02/B1-08/B1-09	46	MZ028363-MZ028408	KJ968190/ KJ968191	98.47-100

		B2-11/B2-14/B2-19 B3-05-B3-07 B3-10/B3-12-B3-14 B4-02/B4-04-B4-35				
Nomeidae	*Cubiceps pauciradiatus*	B2-15/B3-17	2	MZ028414-MZ028415	MF956610	99.52
Scombridae	*Thunnus alalunga*	B2-01/B2-04	2	MZ028345/MZ028346	KP259549/ N893175	100
	Katsuwonus pelamis	B2-05/B3-04	2	MZ028351/MZ028352	MT645988/ T645977	100
Phosichthyidae	*Vinciguerria nimbaria*	B2-08/B4-01/B1-16	3	MZ028357-MZ028359	AP006769	100
Sternoptychidae	*Sternoptyx diaphana*	B2-16/B3-19	2	MZ028416/MZ028417	MT323739	100
Balistidae	*Canthidermis maculata*	B3-08/B3-09	2	MZ028423/MZ028424	AP009206	99.39-99.54

(No.) number of samples,

After editing, we obtained a consensus length of 655 bp for all barcode sequences, none of which was found to contain stop codons, insertions, or deletions. The overall mean nucleotide base frequencies observed in these COI sequences were as follows: T (28.30%), C (29.30%), A (22.90%), and G (19.50%). K2P model analysis of the intraspecific, interspecific, and intergeneric genetic divergences of the samples revealed that intraspecific distances among larval sequences ranged from 0% to 1.61%, with an average distance of 0.72%, and with the largest distance of 1.61% being found in Diaphus mollis. Genetic distances between species ranged from 9.79% to 36.38%, with an average of 25.99%, which is 36-fold higher than the average genetic distance within species. The genetic distance between genera ranged between 11.40% and 39.60%, with an average of 26.30%, whereas that between families varied between 19.70% and 39.60%, with an average of 27.50% (the minimum genetic distance of genera considers only sequences of different species, the minimum genetic distance for families considers only sequences of different genera, and so on).

Table 2 Genetic divergence (percentage, K2P distance) at different taxonomic levels

Comparisons within	Mean (%)	Minimum (%)	Maximum (%)	S.E.*
Species	0.72	0	1.61	0.01
Genus	25.99	9.79	36.38	0.02
Family	26.30	11.40	39.60	0.04
Order	27.50	19.70	39.60	0.02

* Standard error.

The NJ phylogenetic tree obtained in the present study was constructed using the distance method based on an analysis of the COI sequences of collected larvae and reference sequences available in the public domain, the reliability of which was confirmed by bootstrap values (Table 1, Figure 2). The tree showed that individuals of the same species clustered along the same tree branch and could be clearly distinguished from those of other species. This clustering was found to correspond to the genetic distance values obtained, thereby indicating that the species could be effectively distinguished. Moreover, we detected no taxonomic deviation at the species level, and accordingly established that a majority of the examined species could be authenticated based on analyses using the barcoding approach.

Discussion

Accurate identification of larval fish at the species level can be both difficult and time-consuming when relying solely on morphological characters or comparative taxonomy (Azmir et al., 2017; Neira et al., 2015). In this regard, ontogenetic changes in morphology have long been acknowledged as among the major factors hindering the identification of larval fish, given that reliable information on the morphology and developmental series of larvae generally necessitates artificial insemination and long-term rearing (Kawakami et al., 2010). However, the emergence of DNA barcoding represents a critical step in overcoming these obstacles (Collins et al., 2013; Hacker et al. 2021; Krishna Krishnamurthy et al.2012). This technology can contribute to eliminating current over-reliance on the personal abilities and experience of taxonomists inherent in traditional morphological classification and enables the documentation and standardization of species identification (Barik et al., 2020; Xing et al., 2018). It also has the advantages of high efficiency and accuracy and can also be used to identify substantially damaged samples (Wang et al., 2019). To date, the barcoding approach has been successfully applied in the identification of coral reef ichthyoplankton (Hubert et al., 2015), larval fish in the Eastern Atlantic Ocean (Ardura et al., 2016), the larval fish community in the central Red Sea (Patterson et al., 2017), drifting fish eggs in the Yangtze River (Liu et al., 2018), and larval lantern fish in the Gulf of Mexico (Batta-Lona et al., 2019).

In the present study, we employed DNA barcoding to identify the taxonomy of larval fish inhabiting waters surrounding the Hawaiian Islands, successfully barcoding 92 of the 95 collected samples that were assigned to fish in 12 families, 18 genera, and 20 species. Among these, 17 species were identified at the species level, based on a similarity threshold greater 98% in comparisons with database sequences. These results accordingly confirm that DNA barcoding can be successfully applied to identify a majority of the sequenced samples of larval fish at the species level, and thereby contribute significantly to our current understanding of the diversity of larval fish communities within Hawaiian waters. Moreover, we believe it would have been possible to identify all 20 detected species had the reference database been more complete. However, given the lack of an accurate, large, and robust database with reliable reference sequences, we were unable to classify the remaining three at the species level, which could only be assigned identity at the genus level, as *Bolinichthys* sp.*, Kali* sp.*,* and *Parupeneus* sp., respectively. Incomplete databases represent a substantial bottleneck with respect to species identification (Ko et al., 2013; Wang et al., 2019), as has been reported by Ardura et al. (2016) for Eastern Atlantic Ocean fish larvae and Leis (2015) for Indo-Pacific fish larvae. These findings serve to highlight that the success of DNA barcoding for species identification is particularly dependent on the availability of high-quality reference sequences in public sequence libraries such as BOLD or GenBank. Barcoding does, nevertheless, enable the identification of larvae fish when there are sufficient database reference barcodes with accompanying voucher specimens that are taxonomically ascertained (Ardura et al., 2016; Azmir et al., 2017).

The efficacy of species identification based on DNA barcoding is also dependent on both sufficient inter- and intraspecific divergence (Xing et al., 2020b), with larger barcode-related intra- and interspecific differences in genetic distance contributing to the more successful identification of species (Dhar and Ghosh, 2017). In the present study, we used the minimum interspecific and maximum intraspecific divergence to define the barcode gap and found this more efficient than using the means, with both maximum and mean intraspecific distance values (1.61% and 0.72%, respectively) being inferior to interspecific distances (9.79–36.38%, with a mean value of 25.99%), with no overlap. The mean interspecific distance (25.99%) was found to be 36-fold higher than the mean intraspecific distance (0.72%), which is higher than the 29-fold difference reported for the DNA barcoding of Taiwan Strait fishes and the 25-fold difference obtained for Australian marine fishes (Xing et al., 2018; Ward et al., 2005). These result accordingly confirmed that DNA barcoding can be effectively applied in identifying the larval fish in Hawaiian waters.

In the present study, we used the distance method to construct an NJ phylogenetic tree based on COI sequences, the reliability of which was confirmed by bootstrap evaluation (Figure 2). The tree revealed the same topology for the clustering of closely related or well-identified species, which were grouped in the same clade or sister clades. All individuals of larval fish samples grouped into the same specific level compared with the identified target species, and among the 20 species initially detected, 17 fish species were reliably identified at a specific level based on comparisons with the sequences from validated fish species.

Conclusions

In conclusion, the results of this study provide convincing evidence that DNA barcoding can serve as an effective tool for the accurate species identification of larval fish in Hawaiian waters, and could thereby make a valuable contribution to species diversity surveys. Among the 95 larval fish samples collected, 92 were successfully barcoded and identified as belonging to 12 families, 18 genera, and 20 species. Our findings also serve to emphasize the need to obtain a larger number of reference COI barcodes for fish species to enhance the potential utility of barcoding with respect to gaining estimates of larval fish community diversity. The findings of this study enhance our current knowledge regarding larval diversity and dispersal, an understanding of which is considered an essential facet of fisheries management.

Materials And Methods

Sample collection

Sampling was conducted at four stations in Hawaiian waters in October 2019, the locations of which are shown in Figure 1. Larval fish were sampled using a bongo net with a mouth opening of 80 cm diameter and mesh size of 500 μm. Whole specimens were preserved in 95% ethanol during sampling and subsequently transported to the laboratory. The collected larvae were initially separated from the remaining zooplankton under a dissection microscope, photographed, and thereafter sorted into different morphotypes according to their basic morphological characters. The voucher specimens were deposited in the Marine Biological sample Museum at the Third Institute of Oceanography, Ministry of Natural Resources.

DNA extraction, PCR analysis, and sequencing

Total DNA was extracted from individual larvae using a DNeasy Tissue Kit (QIAGEN), with the quality and quantity of the extracted DNA being determined using a N50-Touch spectrophotometer (NanoPhotometer, Germany). Sequences of approximately 655 bp within the 5’ region of the mitochondrial COI gene were amplified using the upstream primers jgLCO1490 TITCIACIAAYCAYAARGAYATTGG and jgHCO2198 TAIACYTCIGGRTGICCRAARAAYCA (Geller et al., 2013). The 25-μL reaction mixtures contained 16.0 of μL ultrapure water, 2.5 μL of 10× PCR buffer, 2.0 μL of $MgCl_2$ (25 mM), 0.5 μL of each dNTP (10 mM), 0.5 μL of each primer (100 nmol), 0.5 μL of Taq DNA polymerase (5 U/μL), and 1 μL of DNA template. The thermal cycling conditions consisted of an initial denaturation step of 2 min at 95 °C, followed by 37 cycles of denaturation (94 °C, 45 s), annealing (48 °C, 30 s), and extension (72 °C, 1 min), with a final extension at 72 °C for 8 min, after which the

reaction mixtures were held at 4 °C. The samples were subsequently run on 1.0% agarose gels for analysis of the amplification products, the sequencing in both directions of which was performed by Sangon Biotech (Shanghai, China).

Data analysis

The SeqMan package of DNAStar software was used to splice the forward and reverse sequences determine for each sample. Specimens were identified by comparing the COI sequences thus obtained with those in the NCBI database using dedicated statistical tools, and we adopted a sequence similarity of at least 98% as a threshold to determine the validity species identification (Armani et al., 2015; Xing et al., 2020b). Subsequently, the sequences were arranged using ClustalW in MEGA6.0 software. Pairwise genetic distances were calculated using the Kimura-2-parameter (K2P) distance model (Kimura,1980) and a neighbour-joining (NJ) tree was constructed with the K2P model. The reliability of tree branching was evaluated based on bootstrap analysis, with support values for tree nodes being obtained from 1 000 repeated samplings.

Abbreviations

COI: Cytochrome c oxidase subunit 1; BLAST: Basic Local Alignment Search Tool; bp: base pair; DNA: Deoxyribonucleic acid; dNTPs: Deoxynucleotide triphosphates; K2P: Kimura-2 parameter model; MtDNA: Mitochondrial DNA; NCBI: National Center for Biotechnology Information; NJ: Neighbor-Joining; PCR: Polymerase Chain Reaction.

Declarations

Ethics approval and consent to participate: Not applicable.

Consent for publication: All authors consent to publication.

Availability of data and material: All data generated or analysed during this study are included in this published article

Competing interests: The authors have no conflict of interest to declare. This manuscript is not submitted elsewhere and is original.

FundingThe Project of Monitoring and Protection of Ecological Environment in the East Pacific Ocean Sponsored by COMRA (No. DY135-E2-5-03)

Scientific Research Foundation of Third Institute of Oceanography, MNR [grant numbers 2017009, 2019015]

Global Change and Air-sea Interaction under contract (No. GASI-01-NPAC-Stsum)

National Natural Science Foundation of China (No. 41406216)

The Marine Biological Sample Museum Upgrade And Expansion (No.GASI-01-02-04)

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Acknowledgments:

We would like to thank the Pacific Remote Islands Marine National Monument/U.S. Fish and Wildlife Service/Department of the Interior for their support, as well as the National Oceanic and Atmospheric Administration.

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Figures

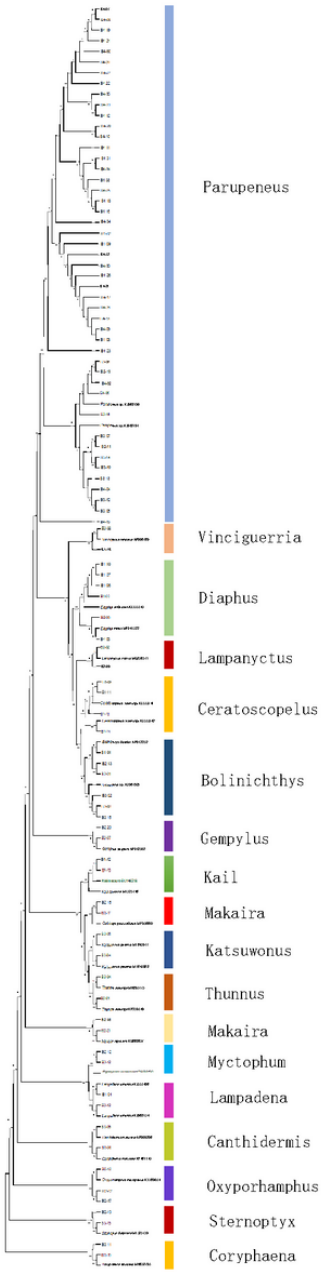

Figure 1

A neighbor-joining tree based on COI sequence and K2P distance analyses.

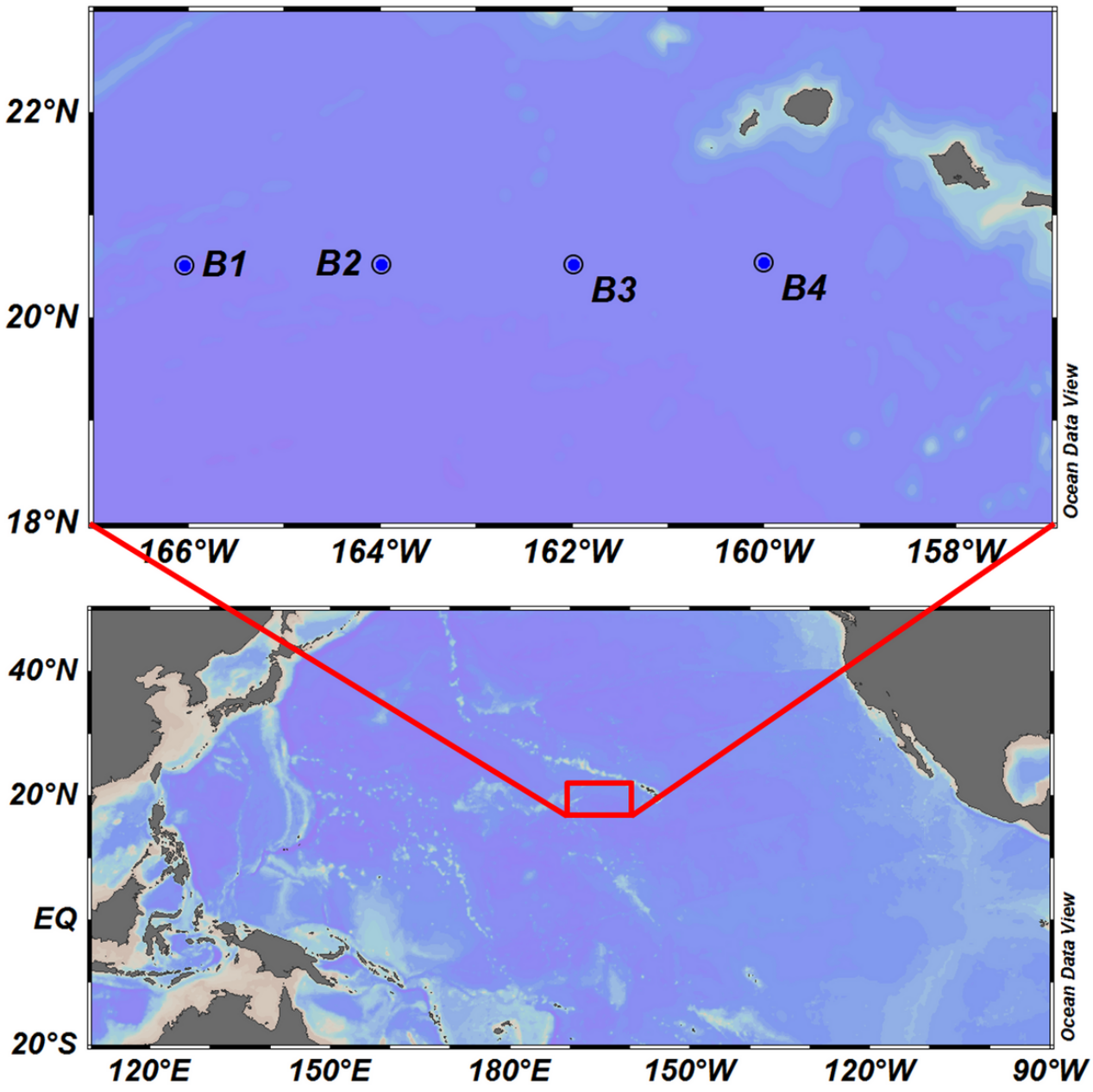

Figure 2

Locations of the sampling stations in Hawaiian waters at which fish larvae were collected.