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Standard Operating Procedure of Gene Sequencing for Jumbo Flying Squid in the South East Pacific

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Molecular techniques are effective methods for studying the genetic variability and phylogenetic relationships among of variety of species (Englbrecht et al., 2000; Whitehead et al., 2003). Understanding and mastering the present genetic diversity and population structure of squid species is the foundation for assessing and managing these fishery resources scientifically and effectively.

In order to detect the population genetic structure of jumbo flying squid in South East Pacific waters, mitochondrial DNA (cytochrome c oxidase subunit I (COI)) is used to obtain the genetic diversity, population structure and phylogenetic relationships among the specimen.

A set of Standard Operating Procedures (SOPs) for field sampling, data recording, storage, shipping and archiving of samples will be developed based on a review of the existing SOPs developed by partner agencies for previous projects. The draft SOP will be presented to the Steering Committee for review prior to finalization and training workshops will be conducted with participating coastal states fisheries officers and researchers.

1 Review of the genetic diversity and population structure research for squid

DNA sequencing techniques have promoted to identify the stock structure in both freshwater and marine fish recently. Compared with protein and nuclear DNA markers, mitochondrial DNA (mtDNA) is playing a critical role in genetic structure, phylogeography, and phylogenetic relationships at various taxonomic levels because of its maternal inheritance, fast evolution and absence from intermolecular genetic recombination (Barrette et al., 1994). Cytochrome c oxidase I , which is known as a protein of mitochondrially encoded cytochrome c oxidase I (MT-CO1), is one of three mtDNA encoded subunits (MT-CO1, MT-CO2, MT-CO3) of respiratory complex IV, which is the third and final enzyme of the electron transport chain of mitochondrial oxidative phosphorylation. Otherwise, the Cytochrome c oxidase I also play a key place in aerobic metabolism (Brown et al., 1992; Garcia-Horsman et al., 1994; Papa et al., 1994). In the mitochondrial genome, it has a faster evolutional loci than the nuclear gene and the evolution rate is moderate, which can directly provide more abundant DNA polymorphism information (Levinson et al., 1987; Nass and Nass, 1963). Therefore, it has been a useful molecular marker for inferring phylogenetic and phylogeographical difference of marine invertebrates (Folmer et al., 1994).

In cephalopods, the molecular marker has been widely used in the study of genetic diversity and population structure for a variety of squid species. The mtDNA COI was one of the most widely used to assess the genetic variation among populations in

previous studies. Lischka et al. employed the mtDNA COI sequences to evaluate the genetic variation of four species of the family Onychoteuthidae in the Sargasso Sea, and the results have provided some valuable evidences for the biodiversity of Onychoteuthidae (Lischka et al., 2018). Furthermore, phylogeographic inferences for populations of *Doryteuthis plei* in the western Atlantic coasts were made using mtDNA COI, indicating the undocumented a sub-populations structure in the Gulf of Mexico (Marı´a et al., 2017). The COI also has been applied to determine the genetic diversity and phylogenetic relationship of five geographical populations of cuttlefish (*Sepiella japonica*) along with Chinese coast (Li et al., 2014). Wakabayashi et al. (2006) used the COI sequence to investigate the relationships of seven ommastrephid squid.

In addition, the Restriction site Associated DNA sequencing (RAD-seq) method has been successfully applied by AZTI (a private non-profit research foundation promoting sustainable use of aquatic resources carried out by an economically-competitive fleet that exercises responsible fishing activities) to examine the genetic diversity and population structure among tuna species, such as the Atlantic mackerel (*Scomber scombrus*) and the Bluefin Tuna (*Thunnus thynnus*), etc. Furthermore, the Diversity Arrays Technology proprietary patented protocols (DArT-seq) have been shown to be applicable for analyzing population level discrimination of Yellowfin, Bigeye and Skipjack tuna.

2 Standard Operating Procedure (SOP)

2.1 Sample collection

Detailed sampling plans for squid in the South East Pacific, for the sampling to be representative and the individuals of jumbo squid to be collected form wider area, the Sampling area are divided into eight subarea named Zone I, Zone II, …, Zone VIII (Table 1 and Figure 1). These subareas cover the coastal waters and the Convention Area, and make sure the jumbo squid samples derived from different habitat. In each Zone, the sampling site is no less than 3 and sample size is no less than 50.

Table 1 Geography extent of the eight subareas

Subarea	Geography
Zone I	2N-10S, EEZ of Ecuador and northern Peru
Zone II	High seas west of Zone I, the same latitude with Zone I
Zone III	10S-20S, EEZ of southern Peru
Zone IV	High seas west of Zone III, the same latitude with Zone III
Zone V	20S-30S, EEZ of northern Chile
Zone VI	High seas west of Zone V, the same latitude with Zone V
Zone VII	20S-40S, EEZ of central Chile
Zone VIII	High seas west of Zone VII, the same latitude with Zone VII

2.2 DNA extraction

Detailed sampling plans for squid in Peruvian waters, including their geographical locations and sample size are given in Table 1. Muscle tissues (5 g) from the tail was taken from each individual and immediately preserved in 95 % ethanol and stored at -20 °C for further processing. The genomic DNA of each individual was extracted from the tail muscles by using a standard phenol/chloroform method (Sambrook et al.,

1989). The quality of DNA was detected on 1.0 % agarose gels prepared in 1 × TAE buffer.

2.3 COI sequence amplification and sequencing

The partial COI (622 bp) (Accession No: AB270944.1) was amplified using the primers (Sense 5′- ATCCCATGCAGGCCCTTCAG-3′; Antisense 5′- GCCTAATGC TCAGAGTATTGGGG-3′). The PCRs were run in a 25 µl volume containing 1 × PCR buffer (50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl, pH 8.3), 0.2 mM dNTPs, 0.5 µM each primer, 100 ng of genomic DNA and 1 U Taq polymerase. The PCR reaction was performed in an initial denaturation of 5 min at 95 °C, followed by 30 cycles of PCR (denaturing at 95 °C for 45 s, annealing at 55-60 °C for 30 s, and extension at 72 °C for 1 min) and a final elongation at 72 °C for 10 min. PCR products were purified by ethanol precipitation and sequences were analyzed using the Next Generation Sequencing (NGS) technology.

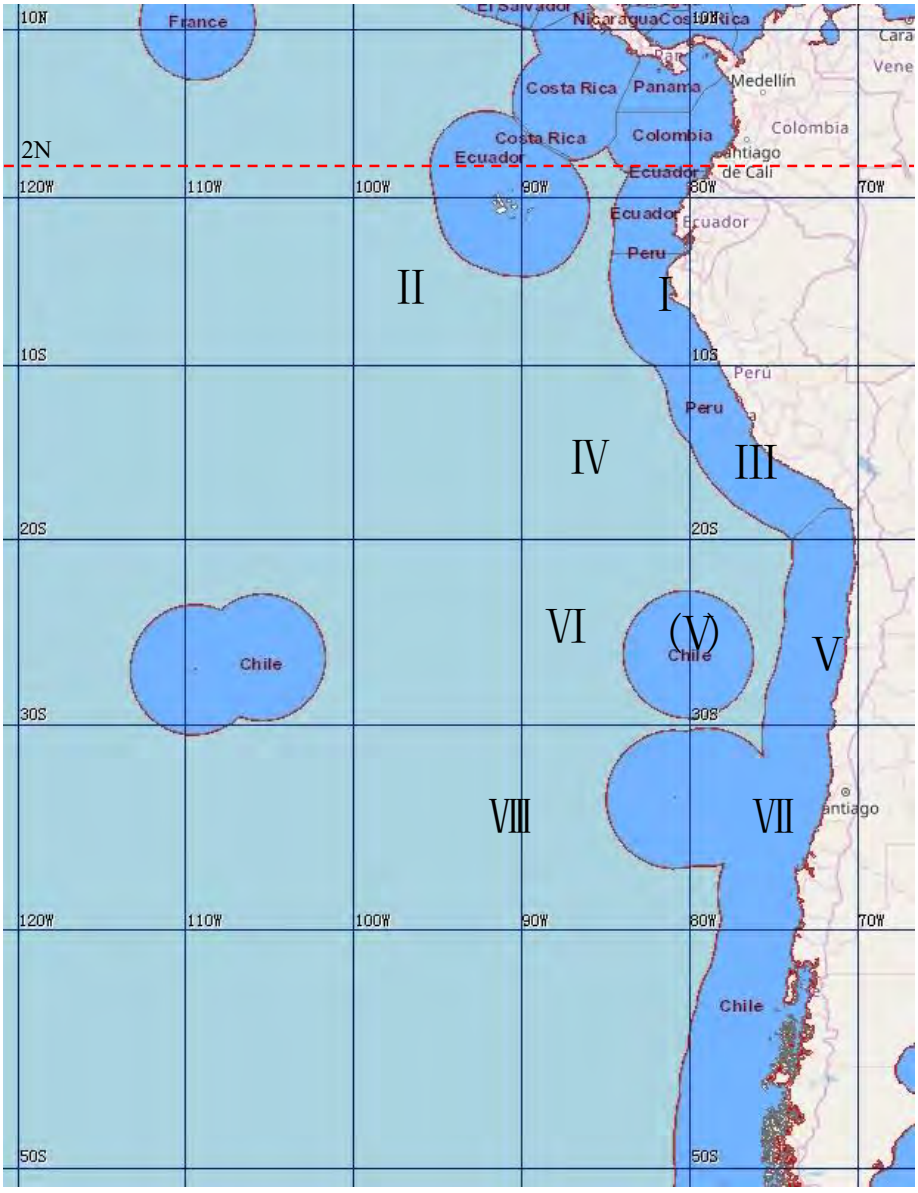

Figure 1 Illustration of the geography extent of the eight subareas

2.4 Data analysis

The sequences of COI were aligned using the CLUSTAL X (Thompson et al., 1997) and manually inspected. In order to detect the variation, the sequences were detected using MEGA version 6.0 (Tamura et al., 2013). The standard genetic diversity indices, such as the number of haplotypes (H), haplotype diversity (Hd), nucleotide diversity (Pi) and gene flow (Nm) were performed with the software DNASP 4.0 (Rozas et al., 2003).

The phylogenetic trees of the haplotypes were constructed based on the neighbour-joining (NJ) method (Saitou and Nei, 1987) and maximum-likelihood (ML) method (Felsenstein, 1981) calculated with parsimony analysis in PAUP*v.4.0b8 (Swofford, 2003). The robustness of statistical support for the phylogenetic hypotheses was determined by a permutation test with 10,000 replicates. Moreover, the software Network 4.0 was used to construct the median joining (MJ) network of the haplotypes. A neutral test was detected by the tests of Tajima's D (Tajima, 1989) and *Fu's Fs* (Fu, 1997), which were calculated using Arlequin 3.5 (Excoffier and Lischer, 2010), and was performed to probe the demographic history and to estimate the population expansion process of *Dosidicus gigas*. The respective P values were obtained using 1000 simulations. In addition, the mismatch distributions were also implemented (Rogers and Harpending, 1992) using the DNASP 4.0 program to verify whether a population had undergone population expansion.

The expansion time was inferred by the equation T = Tau/2u (Rogers and Harpending, 1992), and Tau was acquired from the mismatch distribution model. The value of u (the mutation rate per sequence and per generation) was computed to 2μk. The μ and k represented the mutation rate per nucleotide and the number of nucleotides in the analyzed fragment, respectively. Furthermore, the approximate time of expansion was evaluated via multiplying t by the generation time.

The variance within and among stocks was calculated by analyzing the pairwise F*st* value, which was carried out according to an Analysis of Molecular Variance (AMOVA), as implemented in Arlequin 3.5 (Excoffier and Lischer, 2010). P value was tested using the coalescent simulations (1000 replicates).

The same software such as CLUSTAL X, DNASP 4.0, PAUP*v.4.0b8 and so on shall be used to ensure the consistency of data analysis.

2.5 Data sharing and exchange

We propose Members and CNCPs that participate in the jumbo flying squid fishery to join in the genetic analysis work for this species. To facilitate samples exchange and sharing between Members and CNCPs, the COI sequences of all samples shall be shared with associated information of sampling location and time.

Members and CNCPs that get others' COI sequences of the jumbo flying squid shall analyze data based on methods presented in section 2.3 and exchange their results. Each Member and CNCP should nominate a coordinator to take charge of data sharing and exchange.

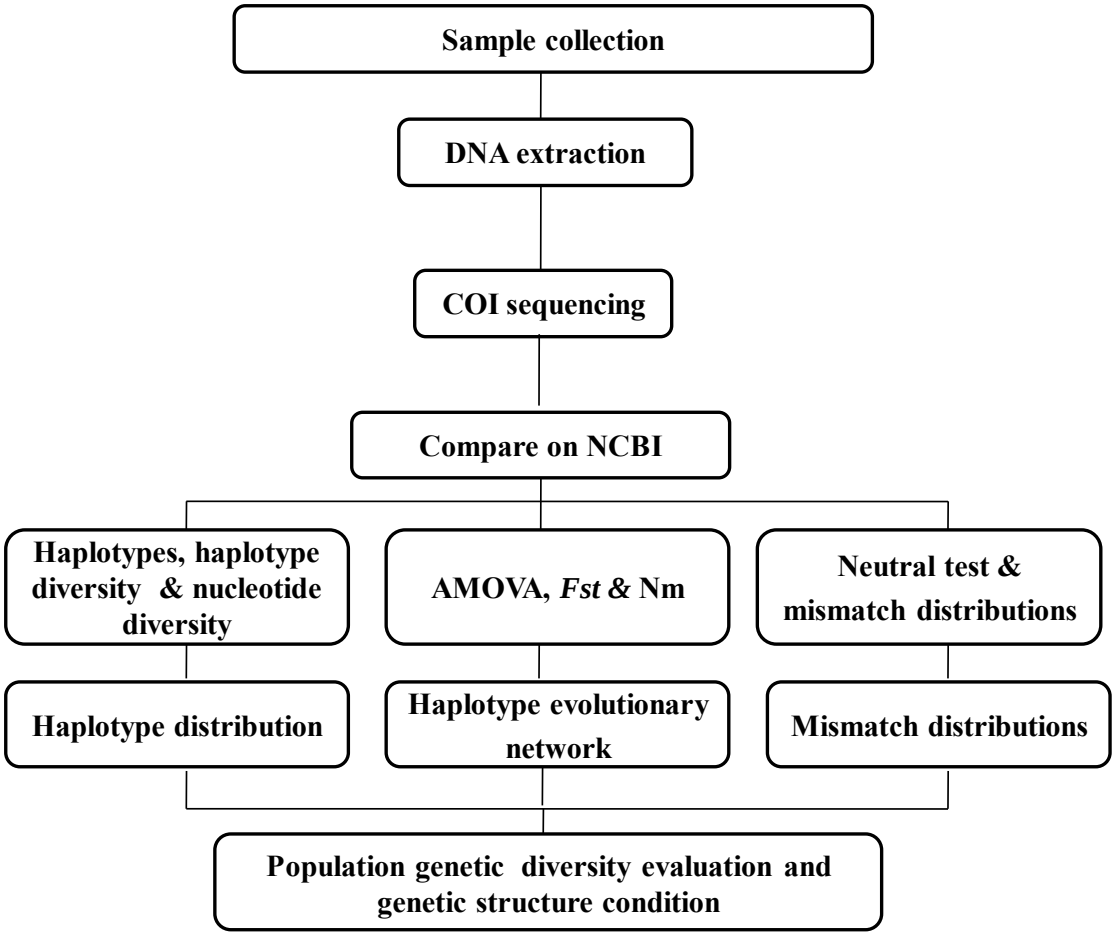

Figure 2 Flowchart genetic analyses for jumbo flying squid

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