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Molecular Identification of Species and the Geographic Origin of Seafood

Michiaki Yamashita,[1]* Atsushi Namikoshi,[2] Jun Iguchi,[2] Yasuharu Takashima,[2] Mohammed Anwar Hossain,[1] Takeshi Yabu[1] and Yumiko Yamashita[1]

[1]*National Research Institute of Fisheries Science Fukuura, Yokohama 236-8648, Japan*

[2]*Food and Agricultural Materials Inspection Center Headquarter Shintoshin, Saitama 330-9731, Japan*

*E-mail: mic@affrc.go.jp

Species and origin identification techniques are required for food labeling regulations to differentiate commercially important fish, shellfish, and seaweed species, because many of the distinguishing morphological features are no longer identifiable after food processing. In Japan, the Law Concerning Standardization and Proper Labeling of Agricultural and Forestry Products (JAS Law) has been establishing food quality labeling standards for foods and beverages. In perishable foods, product names, the production site, and country of origin must appear on the food label. In executing such functions, biochemical and DNA-based genetic techniques for rapid but critical identification of fish species and population have been investigated to determine whether the food labels describe regally correct information.

KEYWORDS species identification; origin identification; food labeling; trace element; mitochondrial DNA; mass peptide mapping

1. Introduction

Rapid identification techniques of species and geographical origin are the current requirement for the purpose of fraudulent labeling prevention and food regulation control with the development of quality assurance systems. Differentiation of commercially important fish and shellfish species of various origins are of importance for the benefit of the product consumers. Identification of species used in preparing processed products is difficult because many of the distinguishing morphological features are no longer visible after processing. As price differences exist among various species and populations in manufacturing seafood products, negative substitution of cheaper materials from fish and shellfish with more

expensive ones in processed products is possible. Such undeclared substitution of the food constitutes in the processed products would not only be in contravention of food legislation but would mislead consumer interests as they choose products based on the labeling information.

In Japan, the Law Concerning Standardization and Proper Labeling of Agricultural and Forestry Products (JAS Law) has been establishing quality labeling standards for foods and beverages, and imposes them on manufacturers and others to assist consumers in selecting goods. In the case of perishable foods, the product name, production site, and country of origin particularly for imported products must be incorporated in the label messages. This rule states the definitions, requirements for consumer notification, product marking, and record-keeping responsibilities of retailers and suppliers with respect to production sites and country of origin for the labeling of raw materials for processed foods. Consequently, for imported marine products, country of origin labeling for processed foods is required. Thus, the prevention of fraudulent labeling of seafood constitutes and their products is a reasonable challenge in line with the maintenance of food regulatory control and development of quality assurance systems. The development of biochemical and DNA-based genetic methods for species and product identification are of practical importance to certify correct food labeling practice presence in the marketplace (Table 1).

2. Species Identification Techniques for Food Labeling

Rapid and authentic species identification techniques are required to detect fraudulent labeling with the view of conferring better utilization of processed foods made by using commercially important fish species. It has recently been reported that more than 200 species are used as raw materials for marine products production in Japan, and closely related species harvested from in other countries are also used for marine products development. Since food labeling of the product name, production site, and country of origin is used for such marine products, analytical methods for examining the

Table 1. Species and origin identification techniques for marine products in Japan

Species and origin	Types of food or tissues used for analysis
mtDNA analysis	
- Japanese eel and Atlantic eel	grilled eel fillet
- Japanese horse mackerel and Atlantic horse mackerel	salted, dried fish
- Pacific mackerel, spotted mackerel, and Atlantic mackerel	salted fish and fermented fish
- Alaska pollack and Pacific cod, and Atlantic cod	seasoned egg
- Japanese sea basses and Nile perch	raw meat
- Red king crab and blue king crab	boiled and frozen-stored meat
- Pacific and Atlantic bluefin tana, and other tuna species	raw meat or frozen-stored meat
- Sea urchin imported from North and South America	frozen-stored gonad
Peptide mass mapping	
- tuna species and skipjack tuna	raw meat, canned and others
Trace elemental analysis	
- Japanese eel from Japan, Taiwan, and China	bone of live fish
- Littleneck clam from Japan, Korea, and China	legs and shells of live shellfish
- Chinook salmon from Japan, Canada, Chili, and Russia	muscle from fresh or frozen fish

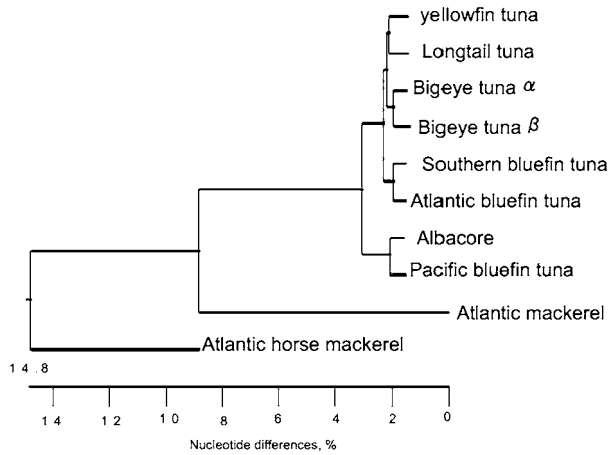

Fig. 1. Molecular phylogenetic tree of tuna species based on complete mtDNA sequences. The complete nucleotide sequences of mitochondrial genome of every tuna species were determined, and their phylogenetic relationship was analyzed using the CRASTAL W program. DNA analysis showed genetic differences and relationship among these tuna species. We used these data for species identification techniques.

authentication of species name and origin used are expected for food regulation and marketing surveys of marine products. Several fish species have been well investigated to develop the analytical methods (Table 1).

2.1. Eel

In recent years, Japan has been occupying the most important eel market in the world, with an annual demand of 120,000 tons. The country annually produces approximately 20,000 tons of Japanese eel (*Anguilla japonica*), which is still insufficient to reach up-to the mark of domestic demand (National Marine Fisheries Service 2005). The difference in the form of the amount between eel demand and national production is subjected to be imported from China and Taiwan as either live fish and processed foods (e.g., grilled eel fillet *kabayaki*). Over the last four decades, however, the juvenile *A. japonica* catch in Asia considerably fell down and prices for juvenile eel rose enormously. In China, to meet eel farm requirements, juvenile *A. anguilla* are imported from Europe. Thus, European eel are produced mainly in China and exported to Japan as a processed food. A PCR-RFLP technique using the mitochondrial cytchrome b gene (*cyt b*) was developed to identify *A. anguilla* produced in China (Wakao *et al.* 1999). This PCR-RFLP technique was used to survey the food labels of commercial eel products to discriminate against processed products made of *A. anguilla* imported from China. Thus, the PCR-RFLP technique can be applied to develop inspection programs for other species to verify correct labeling. Sezaki *et al.* (2005) reported a very simple method to distinguish the above two eel species by PCR with a species-specific primer constructed based on a single nucleotide polymorphism. PCR products show two bands for *A. japonica* and one band for *A. anguilla* in the agarose gel electrophoresis. Furthermore, Itoi *et al.* (2005) detected single-nucleotide polymorphisms with partial sequences of the mitochondrial 16S rRNA gene to distinguish between the two eel species *A. japonica* and *A. anguilla*. They developed species-specific TaqMan minor

Fig. 2. Salted, dried fish of two distinct horse mackerel species. *Trachurus japonicus* is caught in Japan, the annual catch is about 210 thousand tones. Atlantic horse mackerels are imported from European countries in frozen state, annually 43 thousand tones.

groove binder (MGB) probes showing different fluorescence intensity between two eel species.

2.2. Tuna

Price differences exist among the various species of tuna, therefore there are many possibilities for substitution with cheaper species with more expensive one. Such undeclared substitution would not only be in contravention of food legislation by the JAS Law but would mislead consumers' right as they buy the content of products. In line with this, Chow *et al*. (2003) developed a PCR-RFLP method to identify *Thunnus* species using *cyt b* and the flanking regions of the mitochondrial ATPase and CO III genes. This method was successfully used in a market survey for the identification of fresh tuna products by the Ministry of Agriculture, Forestry and Fisheries following the JAS Act of Japan. We determined and compared complete mitochondrial (mt) DNA sequences for tuna species and found various nucleotide substitutions between two pairs of tuna species (Fig. 1). Species-specific DNA sequences could be used to develop novel species identification techniques using DNA array analysis.

2.3. Horse mackerel

Japanese horse mackerel (*Trachurus japonicus*) and Atlantic horse mackerel (*T. trachurus*) are the most commercially important fish among the 14 *Trachurus* species, and Japan occupied its main global market . The *T. japonicus* is caught around Japan, and frozen *T. trachurus* is imported from the Netherlands, Norway, and Ireland as raw materials for salted and dried fish products production (Fig. 2). To characterize and identify mtDNA nucleotide sequence variation in these two *Trachurus* species, the complete mtDNA sequence of *T. trachurus* was determined (Takashima *et al*. 2006). Comparing the determined nucleotide sequences, a PCR-RFLP method was developed to differentiate between these two species (Fig. 3). The primer pair corresponding to ND5 was designed to amplify a 360-bp fragment, and following digestion with *Eco* RI , the PCR product for *T. japonicus* resulted in 93- and 267-bp fragments, whereas *T. trachurus* lacked a restriction site for *Eco* RI. In contrast, after digestion with *Hin* fI, the *T. trachurus* PCR product yielded 44-, 84-, and 232-bp fragments, while the *T. japonicus* product was not digested.

2.4. Alaska pollack and related fishes

A salted and seasoned fish roe product, karashimentaiko is processed from the Alaska pollack (*Theragra chalcogramma*) ovary according to the fair trade competition agreement authorized by the Fair Trade Commission of Japan. However, ovaries of other fish species, such as Pacific cod (*Gadus macrocephalus*), Atlantic cod (*Gadus morhua*), Southern blue whiting (*Micromesistius australis*), and blue whiting (*Micromesistius poutassou*) are mixed in

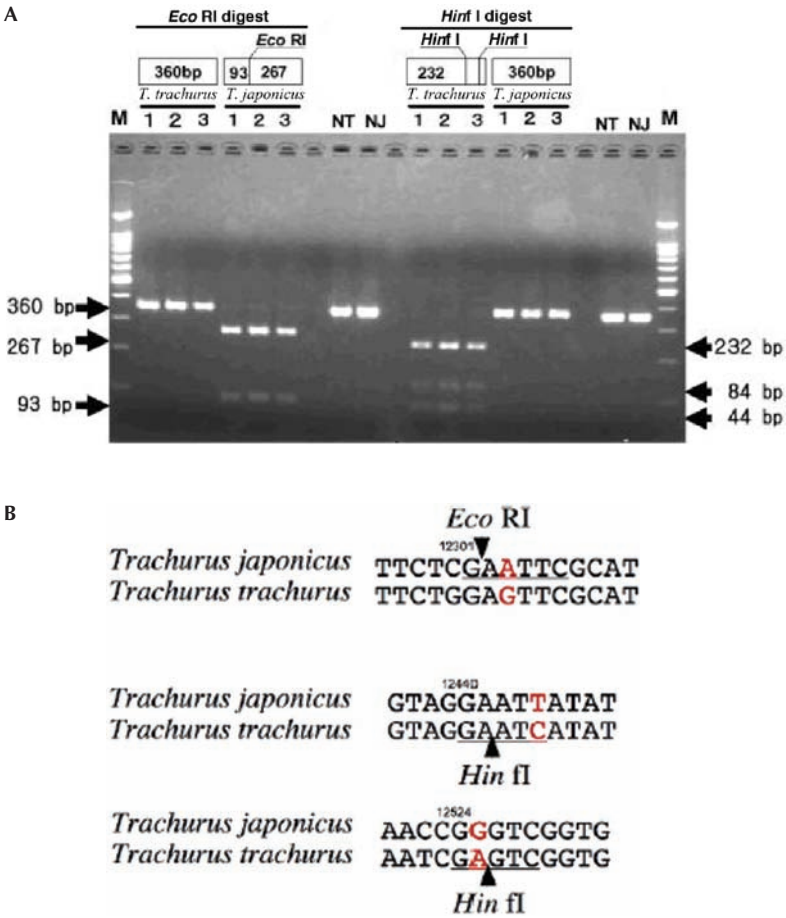

Fig. 3. Species identification techniques for two commercially important horse mackerel species. Three species-specific nucleotide differences at restriction enzyme recognition sites were found in the mtDNA. DNA was extracted by protease digestion and amplified by following polymerase chain reaction with specific DNA primers. After cutting by restriction enzyme of DNA, digested DNA was applied to an agarose gel electrophoresis. Lane M: 100 bp ladder DNA marker; lane 1–3: individual numbers; lane NT: undigested DNA of *T. trachurus*; lane NJ: undigested DNA of *T. japonicus*.

such products. To determine the concerned fish species of these products, we designed a species-specific primer set for Alaska pollack by PCR amplification of a 255-bp fragment encoding the mitochondrial ATP synthase F_o subunit-6 gene. We also designed a species-specific primer set for *Micromesistius* species (*M. australis* and *M. poutassou*) by PCR amplification of a 223-bp fragment encoding the mitochondrial *cyt b*. These PCR -based methods for Alaska pollack and *Micromesistius* species were successfully used for species identification of eight commercially important cod fishes and six fish roe products; the food labeling of raw materials was confirmed to be correct. Therefore, fish species identification techniques for cod roe products were developed using species-specific PCR primers to identify fish species even for a single egg.

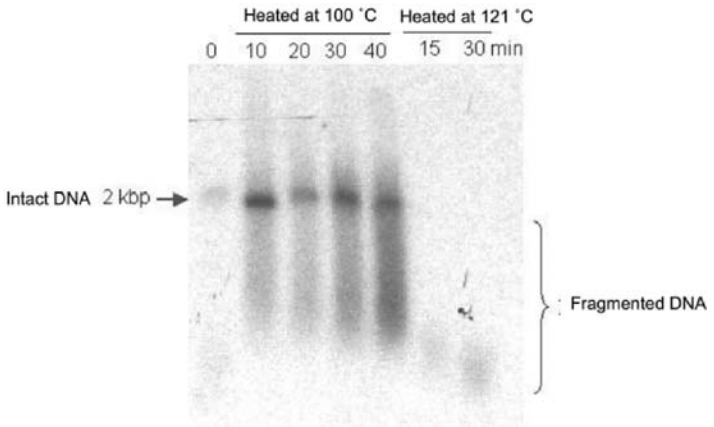

Fig. 4. Southern blotting of 2 kbp DNA fragment in processed fish meat. After the muscles of Japanese horse mackerel were processed at 100°C or 121°C, DNA was extracted, hydrolyzed with *Bam* H1, and then applied to gel electrophoresis. A specific mtDNA fragment DNA was detected with a DNA probe for the *cyt b* gene. The mtDNA was decomposed to small fragments by heating at 121°C for 15–30 min. Therefore, small fragmented DNA is applicable for DNA based analysis.

DNA-based species identification techniques are useful for processed marine products as well as fresh raw materials. The examined degradation patterns of 2 kb-DNA under various heating conditions of food processing, resulted in decomposed smaller sized fragments during heating at 121°C for 15 or 30 minutes (Fig. 4). Therefore, DNA analysis using short mtDNA sequences (<500 bp) was used for steamed, baked, and canned fish products identification. Furthermore, such DNA-based species identification technique using short mtDNA sequences was adapted to identify two related eel species according to the JAS Act of Japan.

3. Elemental and Other Chemical Composition Analyses

Recently, false labeling problems have been encountered in existing eel marketing in Japan. Live Japanese eels imported from Taiwan were illegally sold under Japan originated labeling. Biochemical analytical techniques using multiple trace elemental composition and vitamin K and its metabolites concerning origin identification was practically used to differentiate the geographical origin of these Japanese eels.

Multivariate elemental analysis is increasingly used as a technique to differentiate the geographic origins of plants and wines. Differences existing in specific element composition among regions of origin are important to determine provenance. In the case of fish, otolith (ear stone) chemistry is used as a recorder of time and environmental conditions (Thorrold *et al*. 1998; Campana 1999; Rooker *et al*. 2003). Otolith chemistry is useful for identifying natal origin and assessing the relative contribution of different nursery areas to mixed adult stocks. Thus, in addition to DNA-based species identification techniques, multivariate trace elemental analysis may be useful to determine the geographic origins.

Eels collected from different regions in Japan, Taiwan, and China examined by analyzing the trace and heavy metal contents in the muscles to determine their origin; whether they originated from fish farms or from rivers caught as wild eels (Yamashita *et*

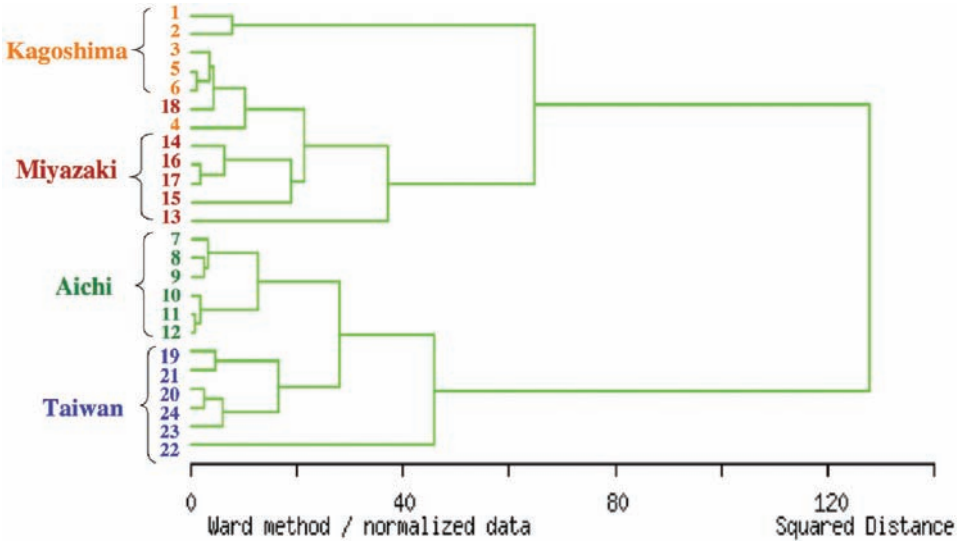

Fig. 5. Origin identification by multivalent trace metal analysis of Japanese eels. Cluster analysis of 12 element contents in eel bone (V, Mn, Co, Cu, Zn, As, Se, Sr, Rh, Cd, Pb, U) showed that the trace elemental patterns were different among distinct geographical origins.

al. 2006). Recently, inductively coupled plasma mass spectrometry (ICP-MS) is useful for the multiple trace elemental analysis. With the ICP-MS detection techniques, a very high sensitivity and selectivity function can be attained for determining rare trace metal concentrations at nM to pM levels. Fish culture systems are very different in Japan, Taiwan, and China. In Japan, most eel farms have tank-based advanced systems using running warm groundwater. In Taiwan and China, eel farms use traditional ponds for aquaculture. Thus, we hope to identify specific trace metal elements accumulate in the muscles and bones of eels derived from groundwater. Rare trace elements taken from the environment, such as uranium, lead, cadmium, and vanadium, are important for determining the origin of eels (Fig. 5).

Menadione (Vitamin K3) is used as a feed additive for fish production in aquaculture (Udagawa 2001; Udagawa and Yamashita 2008). After its intake and metabolism, menadione is converted to menaquinone-3 and this compound is accumulated in the hepatopancreas of eels. Thus, eels grown in tanks in Japan accumulate menaquinone in the hepatopancreus and fat tissues. In contrast, eels are cultured in traditional ponds in Taiwan and southern China. The eels produced in such traditional ponds contain higher levels of phylloquinone and long-chained menaquinone derived from phytoplankton in the culture pond. Therefore, phylloquinone and long-chained menaquinone are detected in fish imported from Taiwan.

The trace element composition of littleneck clam was also examined by Yamashita *et al*. (data unpublished). Japan is occupied an important market for Japanese littleneck clam, with an annual demand of 90,000 tons. However, Japanese production of the species was 37,000 tons in 2003, which is still insufficient to meet domestic demand, and the remainder is imported in live from China, Korea, and North Korea. Due to the recent mislabeling scandals, origin identification techniques based on multivariate analysis of elemental contents have been expected to be applied to determine the

geographic origins (i.e., exporting countries) of clams.

The correlations between the site of origin of Japanese littleneck clams collected from Japan, Korea, and China were examined using trace element content in the muscles and shells of the clams. Distinctive patterns were found in clams originating from Japan, and from Korea and China. The levels of 13 trace elements in muscle tissue varied among the origins. A factorial analysis of the elemental patterns showed that Factor 1 was attributable to cobalt, copper, and strontium levels and Factor 3 to manganese and vanadium levels. Multivariate analysis showed that differences in elemental composition in the muscle between Japanese and imported clams were mainly due to these two factors. In addition, cadmium and arsenic levels observed in the muscles of clams originated from China and Korea were higher than those of clams from Japan, with one exception. Clams from Miyagi, Japan had higher arsenic content compared with those from China and Korea. Therefore, multiple elemental analysis can be used to identify imported clams from China and Korea.

4. Peptide Mass Mapping

DNA and other biological markers are often destroyed in food products during processing, and therefore it is difficult to identify the materials contained in processed food products with DNA-based standard laboratory techniques. Such difficulty running with the DNA-based techniques are limited to apply to processed food products and can be overcome by applying biochemical analysis using mass spectrometry (MS) based on the presence of species-specific amino acid sequences in meat, fish, and plant proteins. In contrast to DNA and other biological markers in processed food which are often decomposed during processing, proteins presence is the sufficient quality in MS.

One possible approach for species identification by protein structure is MS peptide mapping (Fig. 6). Our recent studies represent a method for characterization of species-specific peptide sequences (Yamashita, data unpublished). MS and electrophoresis are powerful tools that can provide microscale analysis of the primary structure of proteins. The coupling of one- and two-dimensional polyacrylamide gel electrophoresis (PAGE) with MS is experiencing a surge of activity, particularly in the areas of protein identification, using database-searching algorithms and characterization of posttranslational modification of proteins. These applications in the field of protein identification employ various types of gel separation and MS techniques that depend on *in situ* chemical or proteolytic digestion of the protein of interest, followed by elution of the digested peptide fragments for MS. This analytical procedure is useful to identify myosin and other muscle proteins in raw materials contained in various marine products such as canned, salted, and dried fish, and *surimi* products (Yamashita *et al*. data unpublished).

5. Future Prospects

The quality of fish and processed products might depend on the quality of the raw materials and geographic origin of their species, which determines their commercial value in the marketplace. Information and databases on the chemical components concerning the determination of food quality, functionality, and safety are very important to assist consumers in selecting foods. Therefore, authentic food labeling regarding species contents and their principle origins should be combined with the labeling information so as to maintain the quality and chemical components of processed food with keeping consumer rights.

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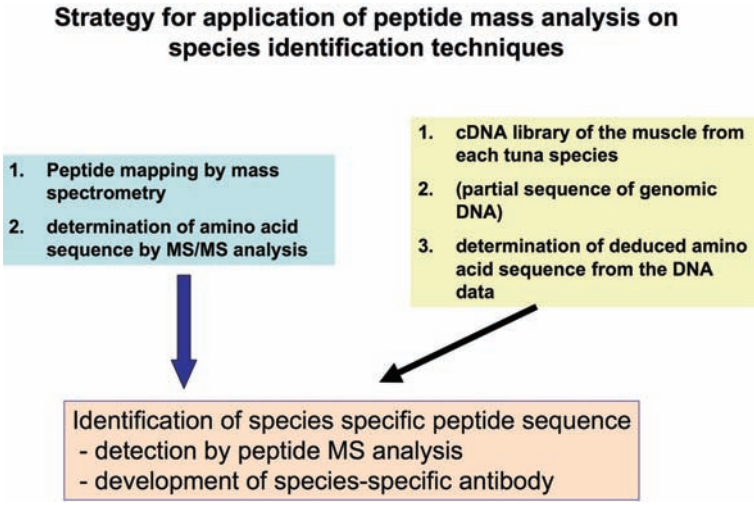

Fig. 6. The scheme of strategy of peptide mass mapping for fish species. Each peptide mass and its amino acid sequence are analyzed by MS/MS analysis after trypsin or other protease digestion of fish meat samples. Nucleotide sequences for the myosin heavy chain and myoglobin are also determined by cDNA and genomic DNA cloning techniques. By comparison with peptide mass and deduced amino acid sequences, species specific amino acid sequence substitutions will be identified. These species specific peptides can be used for species identification by mass spectrometry. A species-specific antibody and enzyme assay kit for species identification will be developed according to the species-specific amino acid residues.

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