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Genetic variability in *Aegorhinus superciliosus* (Coleoptera: Curculionidae) populations in Chilean *Maytenus boaria* (Celastrales: Celastraceae)

Variabilidad genética en poblaciones de *Aegorhinus superciliosus* (Coleoptera: Curculionidae), en *Maytenus boaria* (Celastrales: Celastraceae) de Chile

LUIS HUALA[1], MARCO PAREDES[2], LUIS SALAZAR[3], MARIO ELGUETA[4] y RAMÓN REBOLLEDO[5]

Abstract: The raspberry weevil (*Aegorhinus superciliosus*) is a curculionid native of Chile and part of Argentina. This insect is considered to be a pest to both European hazel (*Corylus avellana*) and blueberry (*Vaccinium corymbosum*) fruit plantations in south-central Chile and has caused considerable economic damage to the local fruit industry. The aim of this study was to evaluate the genetic variability within *A. superciliosus* populations associated with *Maytenus boaria*. Adult *A. superciliosus* specimens were collected from Máfil, Futrono, La Unión and Frutillar populations. Their classification and storage was carried out at the Animal Biotechnology Research Laboratory (LINBA) of the Universidad de La Frontera, Temuco, Chile. Molecular analysis was assessed through ISSR (Inter Simple Sequence Repeats). Results showed high genetic similarities across all studied populations. A positive correlation was observed between genetic variability and geographic distribution. The southernmost populations (Futrono, La Unión and Frutillar) expressed high interspecific genetic similarities, associated with the abundance of *M. boaria,* which allows for genetic flow throughout the studied area. The low level of genetic variability in *A.superciliosus* populations in southern Chile is likely to be correlated with the large number of hosts found in agricultural and forest ecosystems which have the capacity to colonize extensive areas in this part of the country. These conditions contribute to an increase in the genetic flux of *A. superciliosus,* thus transforming this insect into one of the most significant and most harmful blueberry pests in Chile.

Key words: Raspberry weevil, ISSR markers, genetic flow.

Resumen: El cabrito del frambueso (*Aegorhinus superciliosus*), es un curculiónido nativo de Chile y parte de Argentina. En Chile, habita la zona centro-sur y es considerado una plaga importante, ya que afecta a las plantaciones de frutales arbustivos de avellano europeo (*Corylus avellana*) y arándano (*Vaccinium corymbosum*), causando cuantiosos daños económicos a la industria frutícola de la zona. El objetivo de este estudio fue analizar la variabilidad genética de las poblaciones de *A. superciliosus* asociadas a *Maytenus boaria*. Ejemplares adultos de *A. superciliosus* fueron recolectados en las poblaciones de Máfil, Futrono, La Unión y Frutillar. Su clasificación y almacenamiento se realizó en el Laboratorio de Investigación en Biotecnología Animal (LINBA) de la Universidad de La Frontera, Temuco, Chile. El análisis molecular se realizó mediante Marcadores Intermicrosatelitales (ISSR). Los resultados arrojaron una alta similitud génica entre todas las poblaciones de estudio. Se observó una correlación positiva entre la variabilidad genética y la distribución geográfica. Las poblaciones distribuidas más al sur (Futrono- La Unión- Frutillar) presentaron entre si una elevada similitud génica, situación asociada a la abundante presencia de *M. boaria* el cual permite el flujo génico a lo largo del territorio donde se realizó el estudio. El bajo nivel de variabilidad genética en las poblaciones de *A. superciliosus* del sur de Chile, está probablemente asociado al gran número de huéspedes encontrados en los ecosistemas agrícolas y forestales, que tienen la capacidad de colonizar grandes áreas en esta zona del país. Estas condiciones contribuyen a incrementar el flujo génico de *A. superciliosus*, transformando a este insecto en la mayor y más peligrosa plaga de arándanos en Chile.

Palabras clave: Cabrito del frambueso, marcadores ISSR, flujo génico.

Introduction

Raspberry weevil *Aegorhinus superciliosus* (Guérin-Méneville, 1830) (Coleoptera: Curculionidae) is an insect native to Chile and part of Argentina. Its north-to-south distribution in Chile extends from the Maule (35°25'36"S, 71°40'18"W) to the Los Lagos regions (41°28'18"S, 72°56'12"W) (Parra *et al.* 2009).

Exotic fruit trees in this part the country have recorded the highest land use growth over the last decades generating, an agro-ecosystem imbalance, favoring the colonization of *A. superciliosus* (Ellena *et al.* 2012). This insect pest has affected the whole shrub fruit group in south-central Chile and particularly European hazel (*Corylus avellana* L.) and blueberry (*Vaccinium corymbosum* L.) (Quintana *et al.* 2011; Zavala *et al.* 2011; Rebolledo *et al.* 2012).

Most of the damage produced by *A. superciliosus* occurs during its larval stage, as it feeds mainly on roots, causing dwarfism and weakening and during intensive attacks, larvae might possibly cause the death of the plant (Kuschel 1951; Elgueta 1993; Cisternas *et al.* 2000; France *et al.* 2000; Mutis *et al.* 2010; Medel *et al*. 2013; Tampe *et al.* 2016).

Maitén (*Maytenus boaria* Mol.) (Celastrales: Celastraceae) is a native tree from Chile and Argentina (Rodríguez *et al*. 1983), with important morphological variations along its wide distribution, extending in Chile from 29ºS to 49ºS latitude

[1] Ph. D. en Cs. Mención Biología Celular y Molecular Aplicada, Universidad de La Frontera. Francisco Salazar 01145, Temuco, Chile, *luishuala@gmail.com*. [2] Ph. D. en Cs. Mención Biología Celular y Molecular, Universidad de La Frontera. Francisco Salazar 01145. Temuco, Chile, *marco.paredes@ufrontera.cl*. [3] Ph. D. en Farmacia Mención Análisis Clínico, Universidad de La Frontera. Francisco Salazar 01145. Temuco, Chile, *luis.salazar.navarrete@gmail.com*. [4] M. Sc. en Zoología. Museo Nacional de Historia Natural. Santiago, Chile, *mario.elgueta@mnhn.cl*. [5] Ph. D. en Producción y Protección Vegetal, Universidad de La Frontera. Francisco Salazar 01145. Temuco, Chile, *ramon.rebolledo@ufrontera.cl.* Corresponding autor: Ramón Rebolledo, Ph. D. en Producción y Protección Vegetal, Universidad de La Frontera. Francisco Salazar 01145. Temuco, Chile, *ramon.rebolledo@ufrontera.cl*

(from Atacama to the Magallanes regions). For this reason, and in relation to its wide environmental adaptability, this tree has been catalogued as a plastic species (Donoso 1993).

Characterized as a tree without forest used and despite being found in most forest types in Chile, forest-level research in maitén species has not generated interest (Donoso and Wendler 1985). Nonetheless, the medicinal use of its, foliage has prompt interest in chemical studies to prove the anti-inflammatory and antipyretic effect of their glycosidic components (Muñoz *et al.* 1995). Céspedes *et al*. (2000) studied the inhibitory effect of sesquiterpenes in *M. boaria* seeds in photosynthesis and in subsequent studies, the mechanisms associated with insecticidal effects of the same chemical compounds (Céspedes *et al*. 2001).

The large morphological variability found in *A. superciliosus* in its natural distribution has allowed us to consider these as subspecies (Aguilera and Rebolledo 2001). However, it is now estimated that the morphological differences are associated with the color and distribution of the exoskeleton scales (Elgueta 1993).

Taking into consideration the great genetic diversity found among insects species and between populations of the same species (Gadelhak and Enan 2005), the use of molecular tools has allowed for the generation of DNA "fingerprints", used in the identification of individuals, species, subspecies, in genetic rank of populations and in green applications (Korpelainen *et al.* 2007).

The use of ISSR (Inter Simple Sequence Repeats) markers appears to be an efficient tool for the detection of genetic variations (Tikunov *et al.* 2003). This technique also allows for the examination of a large variety of genomic loci, using only one oligonucleotides sequence from 16 to 25 pb making the amplification of areas located between microsatellite DNA sequences, through PCR possible. Microsatellites are distributed through the genome of eukaryotic organisms and are flanked by highly conserved sequences (Chambers and MacAvoy 2000). The polymorphisms can be detected between individuals from the same population, mainly because this analysis is sensitive to the presence or absence of the genomic element recognized for the primer, and to length of the amplified intermediary sequence (Zietkiewicz *et al*. 1994).

A high DNA concentration is not required in the use of ISSR and no previous knowledge in the genome sequence of the studied individual is needed when designing the primers (Joshi *et al.* 2000), leaning on the high grade of polymorphism and huge distribution of microsatellites to detect low levels of differentiation, generating several polymorphic band (Yua *et al*. 2011).

Although *A. superciliosus* is considered as an important species, its biology background focuses mainly in behavior, growth and development (Aguilera and Rebolledo 2001). Studies associated with this curculionid in Chile provide important information regarding its geographic distribution (Kuschel 1951; Vergara *et al.* 2006), morphological description, characteristics of their different stages of development, life cycle, hosts (Parra *et al.* 2009), types of control (Mutis *et al*. 2009) and economic relevance (Aguilera 1994). Carrillo *et al*. (2002) addresses reproductive traits of oviposition and the taxonomic aspects are treated by Elgueta (1993; 2000). Phylogenetic relationship, through its mitogenome sequence, allowed corroborating the monophyletic origin of *A. superciliosus* to the Aterpini tribe (Cabrera and Gaitán 2015).

In spite of the relevant contributions produced by the authors described previously, there is currently there is a lack of information associated with the degree of genetic variability in different populations along their geographic distribution in Chile. With the purpose of detecting possible similarities and differences at genetic level in *A. superciliosus* populations, this research analyzed the genetic variability among populations from southern Chile, associated with *Maytenus boaria*, through ISSR molecular markers.

Materials and methods

Collection of biological material. Adults individuals of *Aegorhinus superciliosus* were manually collected from barks of *M. boaria* in four different areas of Los Rios (39º48'30"S, 73º14'30"W) and Los Lagos (41º28'18"S, 72º56'12"W) regions, in the south of Chile. *Calvertius tuberosus*, was used as control group, due to its large genetic differences with *A. superciliosus*, adult individuals were collected in La Araucanía region (38º54'00"S, 72º42'00"W) (Table 1).

Homogenized tissue and total DNA extraction of *A. superciliosus.* The insects were stored at -80 °C in the Animal Biotechnology Research Laboratory (LINBA), at the Universidad de La Frontera Temuco, Chile, directly after their collection. Individuals were homogenized through disintegration in a previously ultraviolet irradiated (10 min) porcelain mortar and cooled at -80 °C. A total of 1-2 mg of homogenized tissue from each individual was obtained.

DNA extraction from homogenized tissue was carried out through the phenolic extraction protocol adapted from Sambrook and Green (2012), by addition of the previous homogenized individual mix into a 2 ml cell lysis tampon (Tris-HCl 25 mM pH: 8.4, EDTA 2 mM, SDS 1 %) supplemented with 20 µl of proteinase K (10 mg/ml). Incubation was performed in a thermoregulated bath at 55 °C for a minimum of 2 hours. Recovery of 0.7 ml of the lysed in sterile tube (1.5 ml) and application of 0.7 of phenol/chloroform/isoamylic alcohol 25:25:1 (pH: 8.4). Invert to mixed and centrifugation at 10.000 rpm for 10 minutes. Recovery of 0.5 ml from aqueous phase and transfer to a sterile tube. Addition of 0.3 ml of ammonium acetate (10 M) and 1 ml of cold pure ethanol. Incubation of the sample at -20 °C for 20 min. After the incubation period, DNA was extracted through a sterile glass rod to an eppendorf tube (1.5 ml), where it was washed with 1 ml ethanol (75 %) and centrifuged at 5.000 rpm for 1 min. The latter two steps were repeated three consecutive times. To finish, samples were oven drying at 40 °C for 10 min (open lid tube) and DNA sediments suspended with 100 µl of TE buffer.

Table 1. Geographic location of the sampling areas.

Region	City	Coordinates
Los Ríos	Máfil	39°38'41,1"S, 72°57'08,7"W
Los Ríos	Futrono	40°06'49,8"S, 72°27'18,6"W
Los Ríos	La Unión	40°18'16,5"S, 73°04'20,1"W
Los Lagos	Frutillar	41°07'21,5"S, 73°03'33,8"W
Araucanía*	Araucanía	38°54'00"S, 72°42'00"W

* Geographic location of the control group.

Primers design to ISSR markers amplification. Based on the methodology used by Korpelainen *et al*. (2007), 17 ISSR primers were selected (Table 2) and seven were designed in the LINBA (AC-T, CA-G, GA-C, AG-C, AC-C, CA-A, CAG) and synthesized by the Integrated DNA Technologies (IDT), USA. These starters were used to amplify ISSR to PCR from *A. superciliosus* DNA.

Amplification conditions of *A. superciliosus* were adapted and modified according to the protocol described by Pérez de la Torre *et al*. (2012). Terminal amplification conditions were as follows: (a) denaturation for 1 min at 94 °C and for 30 sec at 98 °C (30 cycles); (b) annealing for 30 sec at 55 °C (30 cycles); (c) initial extension for 40 sec at 72 °C (30 cycles) and (d) final extension for 40 sec at 72 °C. The reaction mix was carried out as follows: SapphireAmp Fast PCR Master Mix (2X) 10 µl, DNA (200 ng) 2 µl, Primers ISSRs 2 µl (17 primers) and ultra pure H_2O 6 µl (final volume of 20 µl). The amplification reaction was carried out using a MultiGene Gradient Thermocycler (Labnet International Inc.).

DNA amplification of *A. superciliosus* via PCR using ISSR. A total of 17 primers were evaluated and on the basis of their polymorphic expression patterns, five primers ($[AC]_8$-C, $[GA]_8$-C, $[GA]_9$-A, $[CAA]_5$ y $[GAC]_5$A) were selected. The PCR reaction conditions were assessed as follows: SapphireAmp Fast PCR Master Mix (2X) 10 µl, DNA (200 ng) 2 µl, ISSR primers 2 µl for each and H_2O ultra-pure 6 µl (final volume of 20 µl). The DNA amplification was carried out using a MultiGene Gradient Thermocycler (Labnet International Inc.) (Fig. 1).

Data Analysis. Amplified bands, different and reproducible markers were classified using a value of "1" (presence) and "0" (absence). The data generated were arranged in a binary matrix used for the genetic variability analysis through the Nei's model (Nei 1972) and the construction of a dendrogram by the UPGMA (Bootstrap de 1000) method. Furthermore, the analysis took into account the estimations of the Genetic Differentiation Coefficient (*Gst*); the Genetic Flow Estimation (*Nm*) from *Gst* (McDermott and McDonald 1993) and the Calculation of the Nei's Genetic Diversity Index (*h*) (Nei 1973). Through POPGEN version 1.32 program (Yeh *et al*. 1999).

The Mantel test (Mantel 1967) with 9999 permutations was carried out using the Gene AlEx6 program. And then establishing the relation among the genetic distance and geographic distance, and estimate isolation patterns that could be exists in this population (Peakall and Smouse 2006).

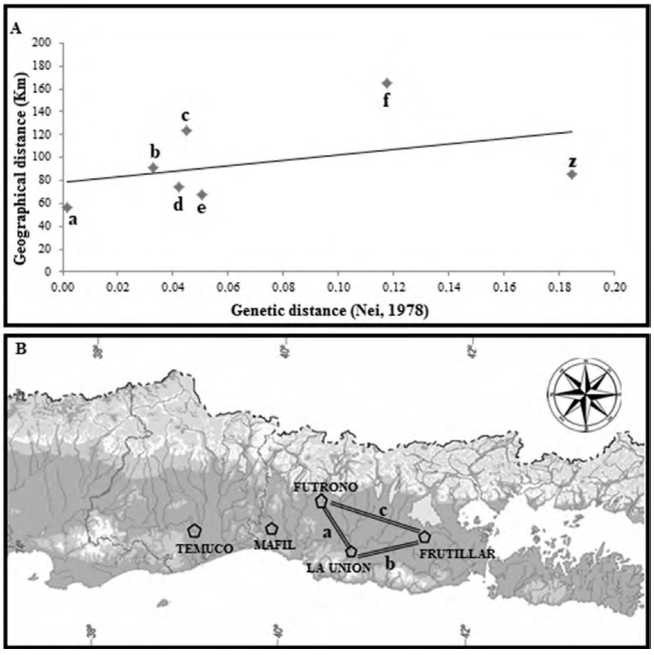


Figure 1. A. Mantel Test. Points: **a.** Futrono-La Unión. **b.** La Unión-Frutillar. **c.** Futrono-Frutillar. **d.** Máfil-La Unión. **e.** Máfil-Futrono. **f.** Máfil-Frutillar. **z.** Máfil-Temuco. **B.** Southern Chile map, with the four populations of *A. superciliosus* and the control population (*C. tuberosus*, Temuco).

Results

The primers selected generated 28 amplified bands 100 % polymorphic, ranging in size between 250 and 1,100 pb (Table 3).

Genetic variability analyses showed substantially high identity values between geographically neighboring populations; Máfil-Futrono (95 %), Futrono-La Unión (99 %), La Unión-Frutillar (97 %). The genetic distance values between populations were: Máfil-Futrono (0.05), Futrono-La Unión (0.002), La Unión-Frutillar (0.03), indicating that the studied populations present practically identical allele frequencies (Fig. 2B).

The average genetic differentiation value between populations was low (Gst = 0.14). The estimated number of individuals migrating between populations in each generation reached an average value of 2.9 (*Nm*). Nei's genetic diversity index (*h*) provided identical values to the populations of Futrono and La Unión (h = 0.11) and lower to those for to Máfil and Frutillar populations (0.07 and 0.08 respectively), which indicates a low genetic variability grade within the studied populations.

A recent cluster that groups Futrono and La Unión populations was possible to be observed through the dendrogram generated by the UPGMA method, collaterally presenting a common ancestor to the Frutillar population. Máfil population, however, is separated from the other three, which would indicate that it apparently derives from a more ancient clade (Fig. 2A).

A direct relationship between genetic and geographic distance matrices (Fig. 1) was provided by the Mantel test. Therefore, the greater geographic distance between

Table 2. Primers used in ISSR analyses.

Primer's name	Sequence	Primer's name	Sequence
AC-T	[AC]8T	CAA	[CAA]5
CA-G	[CA]9G	CA-A	[CA]9A
GA-T	[GA]9T	CAG	[CAG]6
GA-C	[GA]8C	ATG	[ATG]5
GA-A	[GA]9A	GA-C	[GA]9C
AG-T	[AG]8T	AC-G	[AC]9G
AG-C	[AG]8C	CA-T	[CA]9T
AG-G	[AG]9G	GA-C	[GAC]5A
AC-C	[AC]8C		

Table 3. Primers used in ISSR amplification and number of generated bands for each primer.

Primer	Sequency	Total number of bands	Polymorphic Bands (%)	Amplicon's size (pb)
AC-C	[AC]8C	6	100	500-150
CAA	[CAA]5	6	100	1000-400
GAC	[GAC]5A	8	100	1000-300
GA-A	[GA]9A	5	100	1100-400
GA-C	[GA]8C	3	100	1100-250
	TOTAL	28	100	

two populations, smaller their genetic distance. The Máfil population was more independent of the other three as supported by the UPGMA method results. The control group was the farthest when compared to all the studied populations (Table 4).

Discussion

The high genetic identity values observed between the populations of Futrono, La Unión and Frutillar may partly be explained by both the geographic proximity between them and the presence of *M. boaria,* the main host across all the studied territory. The principle of isolation by distance (Wright 1943) the geographically neighbors populations are more similar at the genetic level than the populations that are farthest, phenomenon that is possible to observe within the populations in study.

Southern Chilean landscape (38°54'00"S, 72°42'00"W to 41°28'18" S, 72°56'12" W) is characterized by a continuous vegetation cover along wild and rural areas, paths and roads, where *M. boaria* is a frequent tree (Hoffmann 1998). *Maytenus* trees grow in association with *Rubus ulmifolius* (Schott), *Salix viminalis* (L.), *Rubus idaeus* (L.) species, all of which are cataloged as usual hosts of *A. superciliosus* (Zavala *et al*. 2011). Therefore, all this vegetation plays an important role in the distribution and dispersal of this pest.

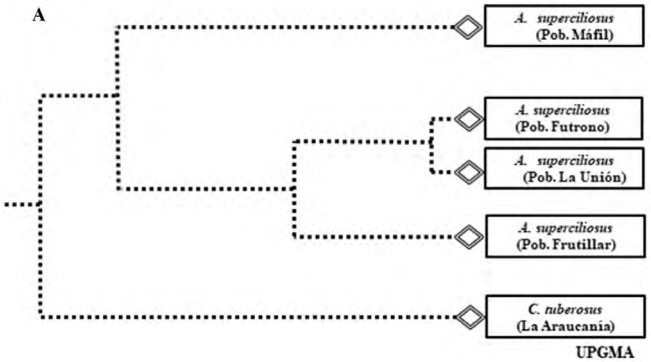
This vegetation cover in the studied area, allowing the genetic flow, thus generating lesser variability among the studied populations, as supported by the low genetic differentiation value of the populations (Gst = 0.14).

The genetic flow estimator Nm provided an average value of 2.9, indicating little genetic differentiation between the studied populations, as also observed by McDermott and McDonald (1993), where the average migrant individuals is sufficient to prevent a substantial differentiation between these populations. Slatkin (1985) proposed that the low genetic distance values are mainly due to the genetic flow existence, which was clearly observed in the *Aegorhinus* populations under study. Therefore, the isolation by distance phenomenon proposed by Wright (1943), in which individuals tend to associate with the ones who are geographically closest, generate low genetic variability in their populations. Mantel test is centered in producing matrices of genetic similarity and geographic distance derived from original data. It is interpretation makes the genetic similarity of neighboring populations clear (López and Olano 2006). The positive correlation found between geographic distribution and genetic variability (Fig. 1A) of neighboring populations (Futrono-La Unión-Frutillar) supports the understanding of the genetic flow and the constant presence of *Maytenus* along the studied territory.

Additionally, Mantel test results not only showed that neighboring populations had a high genetic similarity, but also that geographically distant populations displayed a greater genetic distance between them. This was the case of the Máfil population (the northernmost) in comparison to the populations of Futrono, La Unión and Frutillar (Table 4). Therefore, the positive correlation observed is solid proof to discard chance (Castellano and Balletto 2002).

Table 4. Relationship between genetic and geographic distance (Mantel test).

Points	Between- populations	Genetic distance (Nei, 1978)	Geographic distance (km)
A	Futrono - La Unión*	0.00	57
B	La Unión - Frutillar*	0.03	91
C	Futrono -Frutillar*	0.05	123
D	Máfil - La Unión	0.04	74
E	Máfil -Futrono	0.05	67
F	Máfil - Frutillar	0.12	165
Z	Mafil - Temuco (control)	0.18	86



Población	Máfil	Futrono	La Unión	Frutillar	Araucanía*
Máfil	***	0.95	0.95	0.88	0.83
Futrono	0.05	***	0.99	0.96	0.89
La Unión	0.04	0.002	***	0.97	0.91
Frutillar	0.11	0.05	0.03	***	0.90
Araucanía*	0.18	0.11	0.09	0.10	***

Figure 2. A. UPGMA dendrogram based on genetic distance of Nei (1978) between *A. superciliosus* populations. **B.** Genetic similarity (on the diagonal) and Genetic distances (under the diagonal) (Nei, 1978) on studied populations. (Control: *C. tuberosus*, Araucania region).

The low genetic distance values found in *A. superciliosus* populations in this study (Fig. 2B), are supported by the results obtained by Elgueta (1993). The author describes that the morphological differences observed are correlated with the color and the scales distribution on the exoskeleton. These characteristics may be possibly associated with the age of the adult individuals or with the effect of the high phenotypic plasticity exhibited by the coleopterans, which allows them to adapt to many and different environmental conditions (Moczek 2010).

The genetic distance between the Máfil population (the northenmost) and the Frutillar population (the southernmost) is the greatest compared with the other populations. Two possible explanations have been provided: (a) the distance between these populations (165 km) is the longest among the four under study, (b) the different weather conditions associated with these populations. The Máfil population has the lowest average rainfall in the Los Rios region (1,200 to 1,600 mm per year), in contrast to the Frutillar population (in the Los Lagos region), with an annual average up to 3,000 mm (INE 2015).

Conclusions

The low levels of genetic variability among *A. superciliosus* populations in southern Chile, is probably to be associated to the large number of vegetal hosts found in agricultural and forest ecosystems. These conditions contribute to increase the genetic flux between their populations and they allow that *A. superciliosus* to be considered one of the most important and most harmful blueberry pests in Chile.

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