Impact of life history on genetic variation in *Trifolium* (Fabaceae) estimated by ISSR

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Abstract

Different life history traits affect the genetic structure of plant species. We investigated the impact of habit form on intraspecific genetic variation in ten *Trifolium* species, by randomly sampling ten genotypes of each species using inter-simple sequence repeats (ISSR). Six of eleven primers produced a total of 108 clear and reproducible polymorphic bands. Numbers and levels of polymorphic ISSR bands ranged from 18 and 16.67% in *T. hirtum* to 81 and 75% in *T. hybridum*, and intraspecific Nei's diversity ranged from 0.0599 to 0.2648 in these species. The polymorphic ISSR loci in annual species varied from 16.67 to 52.78% with a mean of 38.90%, and in perennials from 46.30 to 75.00% (mean 58.49%). The results indicate that intraspecific genetic variation in annuals is significantly lower than in perennials ($P < 0.04$, $n = 10$). The ISSR-based UPAGMA dendrogram based on Nei's distances supported the classification of sections proposed by internal transcribed spacer sequence data opposed to morphological characterization. It was confirmed that section Lotoidea is a monophyletic and section Trifolium is a polyphyletic group. The habits form affected the level of genetic diversity in plants.

Key words: annual, clover, habit form, intraspecific genetic variation, perennial, *Trifolium*.
Abbreviations: AMOVA, analysis of molecular variance; ISSR, inter-simple sequence repeats; ITS, internal transcribed spacer; SSR, simple sequence repeat; UPAGMA, unweighted pair group method with arithmetic mean.

Introduction

Several life history traits, including habit form (e.g. annual and perennial) and breeding systems (e.g. selfing and outcrossing) affect the genetic structure and variation in the plant species (Stebbins 1957; Nybom, Bartish 2000). The perennial species are expected to have higher genetic variation than that of annuals, and among the perennials, long-lived species seem to display relatively higher genetic variation than that of short-lived species (Stebbins 1957; Stebbins 1970; Hamrick, Godt 1996). In addition, predominantly-outcrossing species have generally higher genetic diversity than predominantly-selfing species; while species with equally-mixed breeding systems show an intermediate level of genetic variation (Hamrick, Godt 1996; Nybom, Bartish 2000).

The evolution of selfing in angiosperms has been widely studied both theoretically and empirically (e.g. Stebbins 1957; 1970; Barrett et al. 1997; Fishman, Wyatt 1999; Fausto et al. 2001). Botanists have long noticed that most annual angiosperms have predominantly self-pollination systems (Stebbins 1970; Barrett et al. 1997). There are two interpretations for this association. Firstly, the annual plants obtain greater fitness by selfing through 'reproductive assurance', compared to the perennial species, since there is limitation set by pollen reception by the annuals (Stebbins 1997; Fishman, Wyatt 1999; Fausto et al. 2001). Secondly, selfing in the perennial species decreases fitness through inbreeding depression (Morgan et al. 1997). This is why the majority of selfing plant species are annuals while few are perennials (Lloyd 1992; Morgan et al. 1997).

The annual life history is clearly the result of some type of time-limitation supporting the shortened life cycle, which is endorsed by selfing (Snell, Aarssen 2005). Moreover, selfing in annuals has evolved as a result of strong r-selection in ephemeral habitats, leading to either selection for a shorter time to complete the reproductive cycle, or selection for shorter pollination time (Aarssen 2000).

The selfing mating system in flowering plants has often and independently evolved from an outcrossing mating system, for example, more than 60 times in the Solanaceae (Igic et al. 2006; Vekemans et al. 20014). However, ecological and genetic factors support the relative evolutionary advantage of outcrossing vs. selfing (Charlesworth et al. 1990; Porcher, Lande 2005). The transition from outcrossing to selfing is accompanied with major changes in the life form, as from a perennial to annual habit (Bergonzi et al. 2013; Zhou et al. 2013).

The genus *Trifolium* L. (clover, Fabaceae) comprises

about 255 species and includes many annual, short- and long-lived perennials (Zohary 1972; Gillett, Taylor 2001). The breeding systems in clover is very diverse, ranging from self-incompatible outcrossing, obligate and facultative self-pollination, as well as a mixed mode of pollination systems (Zohary, Heller 1984). The genus is widely distributed across the world, with the widest distribution in temperate regions and the highest species diversity reported from the Mediterranean basin and Western North America (Zohary 1972; Zohary, Heller 1984; Gillett, Taylor 2001). In addition, clover includes many forage species widely cultivated in most parts of the world (Gillett, Taylor 2001).

Inter-simple sequence repeat (ISSR) technique is based on simple sequence repeat (SSR)-targeted genotyping technique, where ISSR primers anchor the SSR sites and consequently amplify the neighboring sites. ISSR markers are highly polymorphic at the intraspecific level among genotypes of a given plant species and can be used as powerful markers to estimate interspecific genetic relationship among closely related species (Zietkiewicz et al. 1994; Davila et al. 1998; Moreno et al. 1998; Pasakinskiene et al. 2000). Assessment of the genetic relationship among congeneric species is also important in conservation of the plant genetic resources (Hamrick, Godt 1989; 1996; Nybom, Bartish 2000). In addition, knowledge of the outcrossing rate of a species is useful in breeding programmes and crop improvement (Chowdhury, Sllnkard 1997).

The current work aimed to determine the relationships of both habit form and breeding systems with level of intraspecific genetic variation among ten annual, short- and long-lived perennial *Trifolium* species using ISSR markers. These species have different modes of breeding e.g. selfing, outcrossing and mixed modes. We also determined systematical relationships among the species, and compared the obtained results with those previously reported based on morphological and molecular markers.

Materials and methods

Studied species

There are ten *Trifolium* species distributed in East-Azerbaijan province of Iran, of which *T. suffocatum, T. campestre, T. hirtum*, and *T. nigrescens* are annuals and *T. hybridum, T. ammbiguum, T. pratens, T. montanum, T. tumens*, and *T. medium* are perennials. These species grow in an ecologically wide range of habitats with different edaphic and climatic characteristics including cold and high altitude, saline soils, acidic forest, meadows, and dry areas. The ten *Trifolium* species studied in the current work have very diverse pollination modes.

T. hirtum All. is a self-pollinating species with a selfing rate over 90% (Harding, Tucker 1964; Chowdhury, Sllnkard 1997; Snell, Aarssen 2005). It is well adapted to infertile soils and dry climates in Mediterranean countries and Minor Asia (Snell, Aarssen 2005).

T. nigrescens Viv. is a self-incompatible species, native to the western Asia and the eastern Mediterranean region (Hoveland, Mikkelsen 1965).

T. suffocatum L. is distributed in Macronesia, North Africa, temperate Asia, and Europe.

T. campestre Schreb. is a selfing species native to Eurasia and North Africa, and found in most temperate regions of the world (Zohary, Heller 1984; Snell, Aarssen 2005). This species grows on different habitats including lawns, roadsides, meadows and pastures (Edsall 1985).

T. hybridum L., unlike its name, is a natural true diploid and a self-incompatible outcrossing species. It is distributed in Europe, the Americas and Asia, and adapted to a wide range of environments, different soil texture and low soil pH and low fertility (Pederson 1995; Frame et al. 1998).

T. ambiguum M. Bieb. is self-incompatible species, native to Asia Minor and Caucasus region, and widely introduced in regions of Australasia, North America and other countries, and adapted to a wide range of soil and climatic conditions (Warren et al. 2011).

T. montanum L. is a long-lived perennial and predominantly outcrossing species (Schleuning et al. 2009).

T. pratens L. is a short-lived (two to three years) perennial outcrossing species with a gametophytic self-incompatibility system (Herrmann et al. 2006). This clover species is widely distributed across the world, including north Atlantic and central Europe, Mediterranean, Balkans, Asia Minor, Iran, India, Himalayas, Russia from Arctic south to east Siberia, Caucasus, and the Far East (Gillet 1985).

Both the perennial *T. medium* L., and long-lived perennial *T. tumens* Steven ex M. Bieb. have a Euro-Mediterranean centre of diversity.

Ten individuals of different genotypes from each of ten *Trifolium* species were sampled from different ecological and geographical regions from across North West of Iran, including East-Azerbaijan province for extraction of DNA and determination of the ISSR profile.

DNA extraction and ISSR-PCR profile

Genomic DNA was extracted from seeds and seedlings following Miller (2002). The concentration of DNA samples was estimated by both gel electrophoresis and spectrophotometry, and adjusted to 10 ng mL^{-1}. ISSR-PCR amplification was carried out using Master Mix (CinnaGen PCR MasterKit, Cat. No. PR8251C), added 5 μL of 10 ng template DNA and 1 μL of 100 pm μL^{-1} primer to total volume 25 μL. Several PCR profiles were examined for each of 11 primers, and the best profile producing the most clear, scorable and reproducible bands were selected for each primer (Table 1). Of 11 primers examined, six primers produced the most polymorphic clear and reproducible bands, therefore, these six were selected for further study. All ISSR-PCR products were repeated three to five times to assure reproducibility of the fragments. The PCR products

Table 1. Primer sequences and PCR profiles used in this study for ten *Trifolium* species growing in East Azerbaijan province, Iran

No. of cycles	PCR profile	Primer sequences	Primer codes
1	95 °C for 2 min, 50 °C for 1 min, 72 °C for 30 s	5'-(AC)8T-3'	A
39	95 °C for 30 s, 50 °C for 1 min, 72 °C for 30 s	5'-(AC)8G-3'	B
1	72 °C for 6 min	5'-(AC)8CG-3'	C
1	94 °C for 5 min	5'-(CA)8GC-3'	D
35	94 °C for 30 s, 50 °C for 45 s, 72 °C for 45 s	5'-(AG)10C-3'	E
1	72 °C for 5 min		
1	94 °C for 5 min	5'-(AG)8GC-3'	F
35	94 °C for 30 s, 52 °C for 45 s, 72 °C for 45 s		
1	72 °C for 5 min		

of ISSR were run on 1.5% agarose gels electrophoresis, followed by staining with ethidium bromide. The electrophoretic patterns of the PCR products were recorded digitally using a Gel-Doc 2000 image analysis system (Bio-Rad, Philadelphia, PA, USA).

ISSR data analysis

ISSR fragments were scored as present (1) or absent (0). Then, the data were entered in a binary matrix for Cluster analysis was performed on this binary data using NTSYS-pc (Numerical Taxonomy and Multivariate Analysis System, ver. 2.02). The number and percentage of polymorphic ISSRs loci were obtained for each species. Genetic diversity within each species was estimated using Nei's (1973) and Shanon's (Lewontin, 1972) information indexes (Popgen ver. 1.32).

Relationship among the species was compared using an unweighted pair-group method with arithmetical averages (UPGMA) cluster analysis dendrogram was generated based on a matrix of Nei's distance through the SHAN (sequential, hierarchical, agglomerative and nested clustering program NTSYS-pc). We also used ITS sequences of the ten Trifolium species deposited in GenBank (Ellison et al. 2006) and generated another UPGMA dendrogram using *Medicago sativa* as an outgroup. Then, the resulting dendrograms were compared in order to assess the consistency between results obtained from ISSR and ITS sequence data. In addition, a third UPGMA dendrogram was also constructed using ISSR data for all 100 individual genotypes of the ten studied *Trifolium* species to assess the ability of ISSR in recognizing and clustering of different genotypes of a given species.

Total genetic variation was partitioned into within- and between-species by analysis of molecular variance (AMOVA) using Arlequin ver. 3.11 (Excoffier et al. 1992). The significance level for F-statistics analoges was determined using 1023 bootstrap replicates.

To assess the impact of life history traits on genetic variation in the studied *Trifolium* species under investigation, the levels of intraspecific genetic variation were compared between annual and perennial species using the Mann-Whitney U Test.

Results

Six of eleven primers examined on one hundred individual plants (ten genotypes from each of ten species) produced a total of 108 clear and reproducible polymorphic bands (Fig. 1). Number and proportions of polymorphic ISSR bands ranged respectively from 18 and 16.67% in *T. hirtum* to 81 and 75% in *T. hybridum*. Similarly, intraspecific Nei's genetic diversity varied from 0.06 to 0.27 in *T. hirtum* and *T. hybridum*, respectively (Table 2). The levelsof polymorphic ISSRs loci in the annual *Trifolium* species ranged from 16.67 to 52.78% with mean value of 38.9%, and for the perennial species from 46.3 to 75% with mean value 58.49%. The proportion of polymorphic ISSR loci in the annual *Trifolium* was significantly lower than in the perennial species ($P < 0.04$, $n = 10$, Mann-Whitney U Test, SPSS, 11.5). Similarly, the within-species Nei's genetic diversity differed in the investigated perennial and annual *Trifolium*. This diversity for the four annual species ranged from 0.0559 to 0.1934 with mean value 0.1431, and in six perennial species varied from 0.1746 to 0.2648 with mean

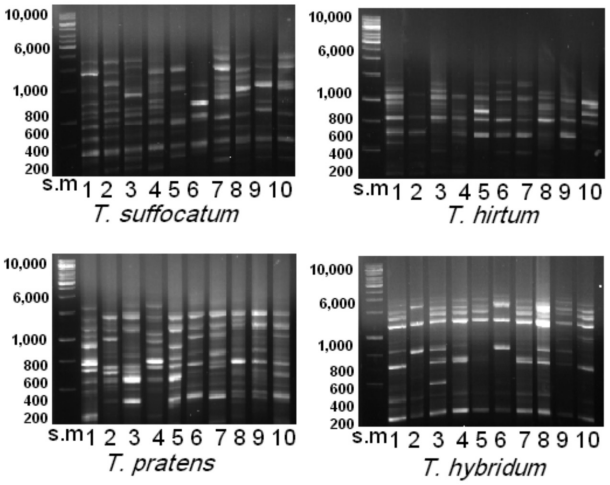

Fig. 1. Samples of ISSR patterns of ten different genotypes of four *Trifolium* species produced by primer A. (Numbers from 1 to 10 in each gel represent ten different genotypes [s.m.= size markers; 200-10000bp)].

Table 2. Primer sequences and PCR profiles used in this study for ten *Trifolium* species growing in East Azerbaijan province, Iran. *, no information on breeding system available

Habit form	Species	Breeding system	No. of polymorphic bands	Polymorphism (%)	Nei's diversity	Shannon diversity
Annual	*T. hirtum*	selfing > 90%	18	16.67	0.0559	0.0855
	T. suffocatum	*	40	37.04	0.1354	0.2020
	T. nigrescens	self-incompatible	53	49.07	0.1878	0.2766
	T. campestre	selfing	57	52.78	0.1934	0.2880
Perennial	*T. tumens*	*	50	46.3	0.1746	0.2584
	T. montanum	outcrossing	57	52.78	0.1917	0.2862
	T. ammbiguum	self-incompatible	61	56.48	0.2082	0.3102
	T. medium	*	62	57.41	0.2140	0.3175
	T. pratens	self-incompatible	68	62.96	0.2346	0.3483
	T. hybridum	self-incompatible	81	75.00	0.2648	0.3976

value 0.2147 (Table 2). The level of genetic diversity in the perennial *Trifolium* was higher than in the annuals, but not significantly ($P < 0.06$, $n = 10$, Mann-Whitney U Test, SPSS, 11.5).

Nei's genetic distance between pairs of species is shown in Table 3. The shortest Nei's genetic distance occurred between *T. pratens* and *T. tumens* (0.053), and the largest distance between *T. ammbiguum* and *T. montanum* (0.338). The UPAGMA dendrogram based on Nei's distances matrix grouped *T. ammbiguum, T. hybridum, T. suffocatum, T. montanum* and *T. nigrescens*, all belonging to section Lotoidea, in one cluster, and the three species of *T. hirtum, T. pratens, T. medium*, all from section Trifolium, in another cluster. *T. tumens* and *T. campestre* were separately located on two different branches removed from the two clusters (Fig. 2 A). *T. hirtum* was closer to the first cluster, despite nesting in the second cluster. It was interesting that in the ISSRs-based UPGMA dendrogram, *T. campestre* occupied a position far distant from the other species. Similarly, in the ITS-based UPGMA dendrogram, the species *T. ammbiguum, T. hybridum, T. suffocatum, T. montanum* and *T. nigrescens* belonging to section Lotoidea nested in one cluster, and the species *T. hirtum, T. pratens, T. medium* from section Trifolium in another cluster. *T. tumens* (section Vesicaria) occupied an intermediate position between the Lotoidea and Trifolium sections, and *T. campestre* (section Chronosimimum) was located in a position as an outlier (Fig. 2 B).

The third ISSR-based UPGMA dendrogram conducted for all 100 individual genotypes belonging to ten *Trifolium* species indicated that all genotypes of a given species grouped together in species clusters (Fig. 3).

Discussion

The results of the current study showed that in *Trifolium*, the perennial species do have higher genetic diversity than the annuals. This can be attributed to the breeding systems, since the annual *Trifolium* species studied are predominantly selfing while the perennials are predominantly outcrossing. In general, the annual plant species predominantly have a selfing breeding system (Stebbins 1970; Snell, Aarssen 2005), which consequently results in a lower level of within-species genetic diversity (Hamrick, Godt 1996; Nybom, Bartish 2000). Annuals can benefit much more from selfing than outcrossing because there is high limitation of outcrossed pollen, i.e. selfing can guarantee reproductive success in annuals, a hypothesis

Table 3. Genetic distance matrix between pairs of *Trifolium* species on the basis of Nei's Distance using ISSRs data

Code	Species	1	2	3	4	5	6	7	8	9	10
1	*T. ambiguum*	0.000									
2	*T. montanum*	0.338	0.000								
3	*T. hybridum*	0.195	0.290	0.000							
4	*T. nigrescens*	0.214	0.279	0.177	0.000						
5	*T. suffocatum*	0.308	0.298	0.173	0.162	0.000					
6	*T. pratense*	0.180	0.351	0.139	0.114	0.181	0.000				
7	*T. medium*	0.215	0.375	0.181	0.187	0.268	0.138	0.000			
8	*T. tumens*	0.178	0.310	0.188	0.105	0.157	0.053	0.169	0.000		
9	*T. campestre*	0.156	0.323	0.132	0.131	0.184	0.101	0.187	0.100	0.000	
10	*T. hirtum*	0.117	0.320	0.155	0.138	0.206	0.114	0.193	0.122	0.098	0.000

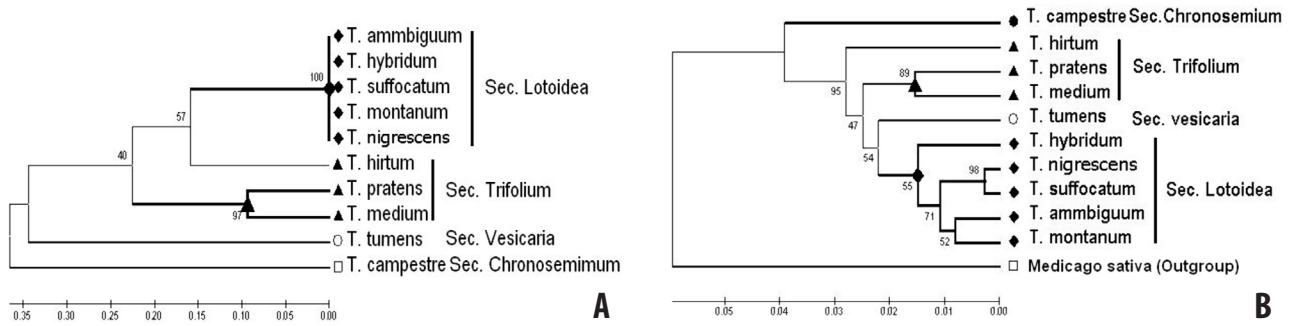

Fig. 2. UPGMA dendrograms showing relationship among ten *Trifolium* species distributed in East-Azerbaijan, Iran. A, Nei' distance-based tree obtained from ISSRs data. B, ITS sequences -based tree constructed on the basis of the sequences obtained from GenBank. Numbers on branches show bootstrap value from 1000 replications..

known as 'reproductive assurance' (Stebbins 1957; Fishman, Wyatt 1999; Fausto et al. 2001). Furthermore, based on the 'time-limitation' hypothesis (Aarssen, 2000; Snell, Aarssen 2005), both selfing and the annual life cycle are concurrent products of strong 'r-selection' associated with high density-independent mortality risk in ephemeral habitats with a strictly limited period of time available to complete the life cycle. Both the reproductive assurance hypothesis (Stebbins 1957; Fishman, Wyatt 1999; Fausto et al. 2001) and the time-limitation hypothesis (Aarssen 2000; Snell, Aarssen 2005) explain the fitness advantage for annuals through selfing. Based on the former hypothesis, outcrossed pollen is not available due to lack of pollinators or mates, while in the latter hypothesis outcrossed pollen is available but arrives too late to develop viable seeds (Snell, Aarssen 2005). Moreover, characters such as small-sized flowers and short plant height in annual species e.g. *T. suffocatum*, could also promote selfing, because, compared to tall plants, shorter plants attract relatively less pollinators and consequently might benefit much less from outcrossing (Donnelly et al. 1998; Lortie, Aarssen 1999). The high levels of genetic variation observed in the studied perennial species of *Trifolium* resulted from a high outcrossing rate (and avoiding selfing), as selfing in perennials can increase the inbreeding depression (Charlesworth, Charlesworth 1987; Lloyd 1992), which in turn results in reproductive failure (Charlesworth, Charlesworth 1990). Moreover, perennial species have higher potential long-range gene movement, which provides an opportunity for outcrossing (Nybom, Bartish 2000).

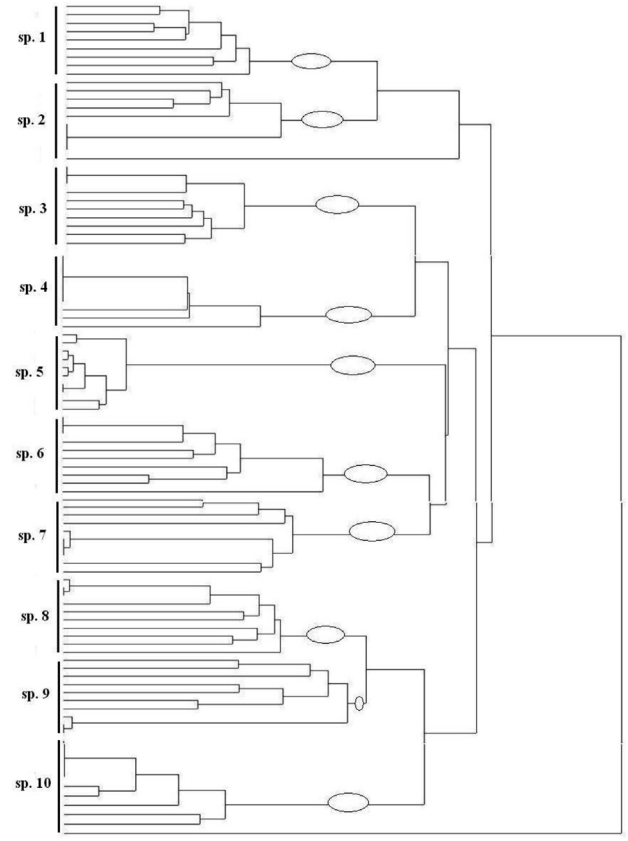

Fig. 2. ISSR-based UPGMA dendrogram conducted for hundred genotypes belonging to ten *Trifolium* species. The species code: 1, *T. ambiguum*; 2, *T. hirtum*; 3, *T. hybridum*; 4, *T. suffocatum*; 5, *T. montanum*; 6, *T. nigrescens*; 7, *T. campestre*; 8, *T. pretense*; 9, *T. tumens*; 10, *T. medium*. The undermost branch is *Medicago sativa* used as outgroup.

There have been some inconsistencies between classification of *Trifolium* species based on morphological characters (Zohary, Heller 1984) and molecular data based on nuclear ribosomal DNA internal transcribed spacer (ITS) and chloroplast *trn*L intron sequences (Ellison et al. 2006). Our results supported classification of the sections proposed by ITS sequence data (Ellison et al. 2006) as opposed to the morphological classification (Zohary, Heller 1984) in showing section Lotoidea as a monophyletic group and section Trifolium as a polyphyletic group, because *T. hirtum* from the latter section shares a progenitor with species from sections e.g. Lotoidea. Our results were also consistent with those obtained from ITS sequence data in showing *T. campestre* as the most distant species to all other species studied. In this study, the ISSR data grouped the *Trifolium* species into their respective sections, supporting the use of these markers at the 'section' level. In addition, the ISSR markers were also capable of grouping all different genotypes of a given species in separate

species clusters despite higher evolutionary rates of these markers, indicating the applicability of the markers in the recognition of plant genotypes.

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