

# Spore ornamentation of *Haplosporidium hinei* n. sp. (Haplosporidia) in pearl oysters *Pinctada maxima* (Jameson, 1901)

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## SUMMARY

An infection of pearl oysters, *Pinctada maxima*, attributed to a *Haplosporidium* sp. by Hine and Thorne (1998) has been detected on 3 occasions and is considered to represent a serious concern to the pearling industry in Australia. The spore ornamentation of the parasite was determined by scanning electron microscopy and transmission electron microscopy. Spores of the parasite were pleomorphic, or elongated  $3.5\text{--}4\ \mu\text{m} \times 2.5\text{--}3.0\ \mu\text{m}$  in size. Two filaments were wound around the spore and originated from 2 'knob-like' posterior thickenings. Both filaments passed up one side of the spore together until just below the operculum whereupon each split and passed obliquely under the lip of the opercula lid. Each filament wrapped around the spore 4 times. The posterior thickenings seem to appear late in the development of the spore and were composed of spore wall material. A second set of branching tubular filaments composed of a different material was observed on the spore body although not on mature spores possessing a 'knob-like' posterior thickening. The ornamentation on the spores of the pearl oyster parasite was unique amongst described haplosporidian species where spore ornamentation is known. The parasite is named in this manuscript as *Haplosporidium hinei* n. sp.

Key words: Haplosporidia, pearl oyster, *Pinctada maxima*, *Haplosporidium hinei* n. sp., *Minchinia*, parasite, SEM, aquaculture.

## INTRODUCTION

The Australian pearl oyster industry is the world's largest producer of the prized south sea pearls that come from the silver lipped pearl oyster *Pinctada maxima* (Jameson, 1901). The industry has an annual export value of nearly \$A300 million with the product being exported primarily to Japan, the United States of America, Hong Kong and Europe (ABARE, 2007). The industry relies on wild-caught adult oysters but has in recent years moved to supplement this production with hatchery raised spat.

An infection of pearl oysters, *P. maxima*, attributed to a *Haplosporidium* sp. by Hine and Thorne (1998) has been detected on 3 occasions (Jones and Creeper, 2006) and is considered to represent a serious concern to the pearling industry (Humphrey and Norton, 2005; Jones and Creeper, 2006). Numerous haplosporidian pathogens have been found in different marine invertebrate species

(Perkins, 2000). Several species including *Haplosporidium nelsoni*, *Bonamia ostreae* and *Bonamia exitiosa* have been associated with epizootic mortalities of commercially important molluscs (Carnegie, 2005). A haplosporidian parasite has also been described infecting rock oysters (*Saccostrea cucullata*) on the same coastline that contains the majority of the Australian pearl industry (Bearham *et al.* 2007; Hine and Thorne, 2002).

The morphology and origin of spore ornamentation, variously termed wrappings, extensions, filaments or tails, is the principal taxonomic feature used to distinguish species and genera within the phylum Haplosporidia. However, the spore ornamentation is not known for many haplosporidian species and this makes species differentiation and genus assignment difficult (Burreson and Reece, 2006). It is likely that the taxonomy of the Haplosporidia will remain confused until spore ornamentation is characterized for a large number of species.

Currently, the most common method to separate the genera *Haplosporidium* and *Bonamia* from the *Minchinia* is based on the origin of the spore ornamentation, from either the spore wall in the case of *Haplosporidium* and *Bonamia*, or from the epispore

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cytoplasm in the *Minchinia* (Azevedo *et al.* 1999; Burreson, 2001; Hine and Thorne, 1998, 2002; Ormieres, 1980). The spore ornamentation from only one species of the *Bonamia* has been characterized. That species *Bonamia perspora*, has been found to possess strap like projections derived from the spore wall (Carnegie *et al.* 2006). The emphasis on spore ornamentation in the taxonomy of the Haplosporidia means it is an important characteristic to be described.

The research presented here describes the spore ornamentation of a haplosporidian in pearl oysters using both scanning and transmission electron microscopy and supports separate species status and assignment to the genus *Haplosporidium*. The parasite is named in this manuscript as *Haplosporidium hinei* n. sp.

#### MATERIALS AND METHODS

Archived formalin-fixed, paraffin embedded pearl oyster tissues infected with *Haplosporidium hinei* n. sp. were obtained from the Western Australian Department of Fisheries and were fixed whole in 10% formalin made up with seawater. This material had been collected from Carnarvon, Western Australia as part of a routine disease clearance prior to transport (Hine and Thorne, 1998). Material specifically fixed for electron microscopy was not available. Sections were processed and stained for histological examination with haematoxylin and eosin. Briefly, unstained sections were cut at a thickness of 4  $\mu\text{m}$  and placed on slides. Sections were dewaxed with xylene and rehydrated in an ethanol series (2 changes for 2 min each). The sections were placed in haematoxylin for 5 min, rinsed in water and then placed in Scott's tap water substitute until blue. The slides were again rinsed in water followed by 95% ethanol (1 change for 30 sec). The sections were stained with 1% eosin for 30 sec and finally dehydrated in 95% ethanol (2 changes 30 sec each), absolute ethanol (2 changes 30 sec each) and xylene (3 changes 30 sec each). Ornamentation on the spores of *H. hinei* n. sp. was assessed through light microscopy, transmission electron microscopy (TEM) and scanning electron microscopy (SEM).

Paraffin-embedded tissue was prepared for TEM by dewaxing in xylene overnight, hydrating through 3 changes of 100, 90 and 70% ethanol. A 1 mm<sup>3</sup> cube of digestive gland tissue was dissected in a puddle of 5% Sorensen's phosphate-buffered glutaraldehyde. The sections were then placed in 5% glutaraldehyde overnight. The samples were then washed in 0.07 M Sorensen's phosphate buffer. The tissues were immersed in Dalton's Chrome Osmic Acid for 60–90 min at 4 °C. The samples were then dehydrated through an ethanol series. Following this, the tissue was immersed in Propylene Oxide, a 60:40 Propylene oxide/Epon mixture and finally

pure 'Epon' overnight. Embedded tissues were sectioned with an mU3/C (Reichert, Austria) ultramicrotome and stained with uranyl acetate. Analysis was performed with a Philips CM100 Bio TEM.

For SEM, infected oyster tissue was removed from the paraffin block and deparaffinized in xylene for 3 days. The tissue was rehydrated through a graded series of ethanol (100%, 90%, 70%, 50% and 30%). The tissue was then sonicated in pure water. A puddle of the resulting suspension was placed onto six 12 mm diameter cover-glasses coated with poly-L-lysine; spores were allowed to settle for 1 h in a moist chamber. The cover-glasses were then dipped in water to remove the excess material and dehydrated through an ethanol series. The affixed spores were then subjected to critical-point drying in liquid CO<sub>2</sub> and coating with gold:palladium. Characterization of the spore ornamentation was based on observations of 26 spores with a Zeiss Leo 435vp SEM.

#### RESULTS

##### *Light microscopy*

Light microscopy of the sections revealed large numbers of pre-sporulation and sporulation stages of the parasite in the connective tissue surrounding the digestive gland and within the digestive diverticulae (Fig. 1). Mature spores with a yellow refractile wall enclosing an eosinophilic sporoplasm were observed. Spore ornamentation was not apparent with light microscopy and parasites were not observed in the epithelia.

##### *Electron microscopy*

Although the fixative used for the study was sub-optimal for electron microscopy, it was sufficient to permit observation of the spore wall and its ornamentation. The fixed spores of *H. hinei* n. sp. were pleomorphic, or elongated 3.5  $\mu\text{m}$ –4  $\mu\text{m}$   $\times$  2.5  $\mu\text{m}$ –3.0  $\mu\text{m}$  in size (Fig. 2). The sporoplasm nucleus was usually basal, but sometimes equatorial and measured 0.8  $\mu\text{m}$ –1.3  $\mu\text{m}$  in diameter (mean = 1.03  $\mu\text{m}$ ). The operculum was situated in the apical zone of the wall and consisted of a circular lid of 1.9  $\mu\text{m}$  to 2.6  $\mu\text{m}$  (mean = 2.24  $\mu\text{m}$ ) diameter (Fig. 2). The lid of the operculum was about 7.2 nm thick and was connected to the spore wall by a hinge. Microfilaments 45 nm–75 nm (mean = 60.72 nm) long and 28.5–34.3 nm (mean = 31.25 nm) wide were also observed on the surface of the spore wall (Fig. 2D). The microfilaments were composed of spore wall material and had focal distribution but were not confined to any particular section of spore wall. They were observed in both immature and mature spore stages.

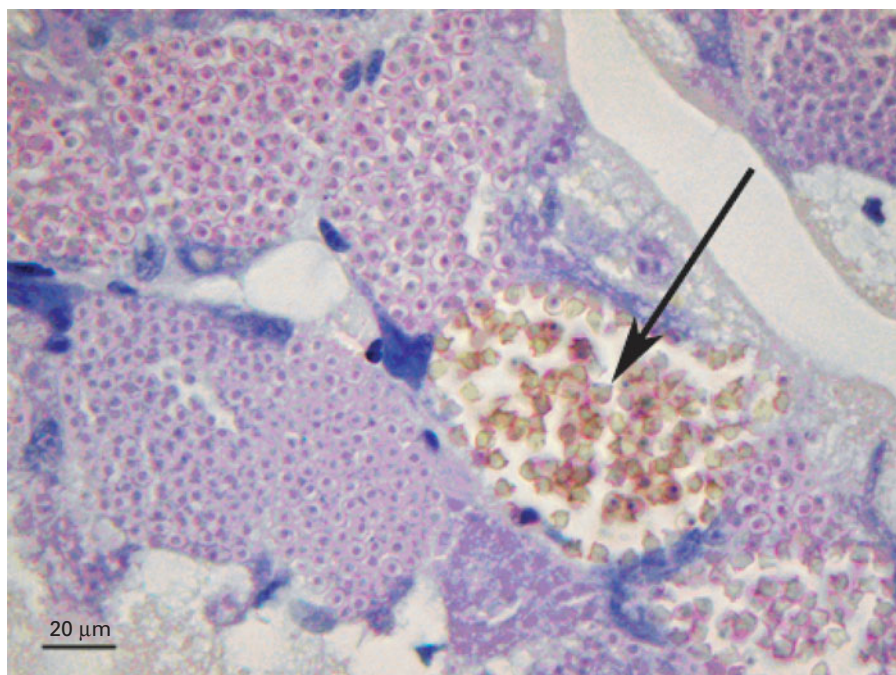


Fig. 1. Pearl oyster digestive gland containing large numbers of the pre-sporulation and sporulation stages of *Haplosporidium hinei* n. sp. in a haematoxylin-eosin stained section. Arrow indicates the yellow refractile mature spores.

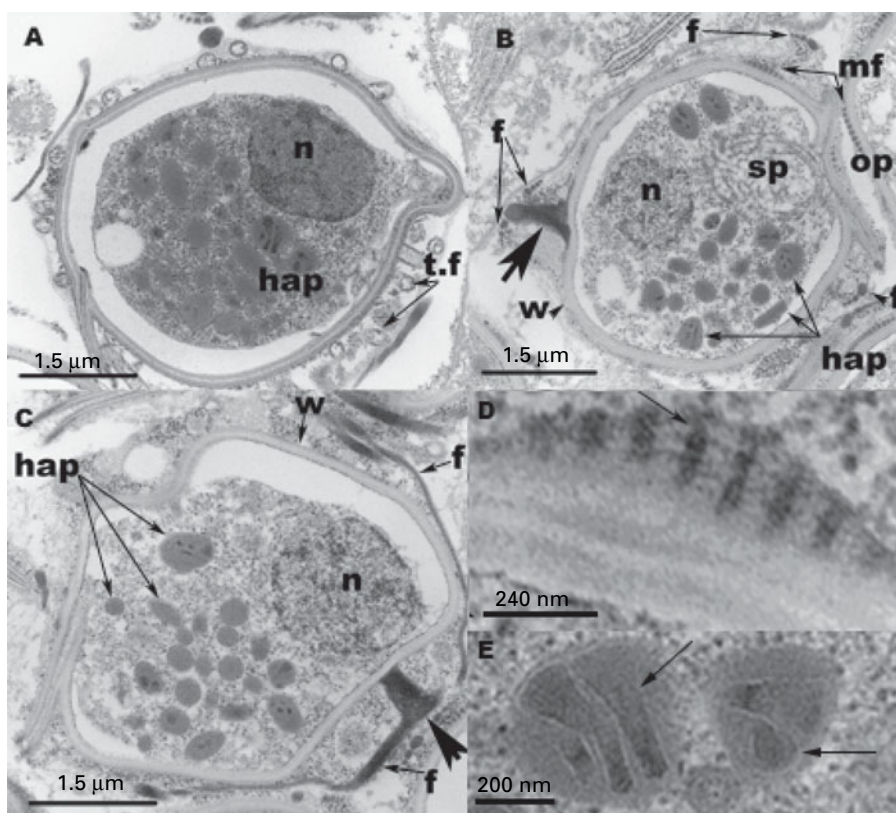


Fig. 2. Transmission electron micrographs (TEM) of mature spores of *Haplosporidium hinei* n. sp. infecting the pearl oyster *Pinctada maxima*. Haplosporosomes (hap) with internal membranes are also present in each photograph. n refers to the basal nucleus, w refers to spore wall, sp refers to the spherule, op refers to the operculum. (A) Indicates the posterior knob and tubular filaments (tf). Tubular filaments are not present in mature spores possessing a knob. (B) Shows the posterior knob and attached spore wall filaments (f) in cross-section. (C) Indicates the spore wall filaments (f) and posterior knob in longitudinal section. (D) A higher magnification view of the microfilaments (arrow). (E) A higher magnification view of the haplosporosomes containing the axie-shaped internal membrane structures.



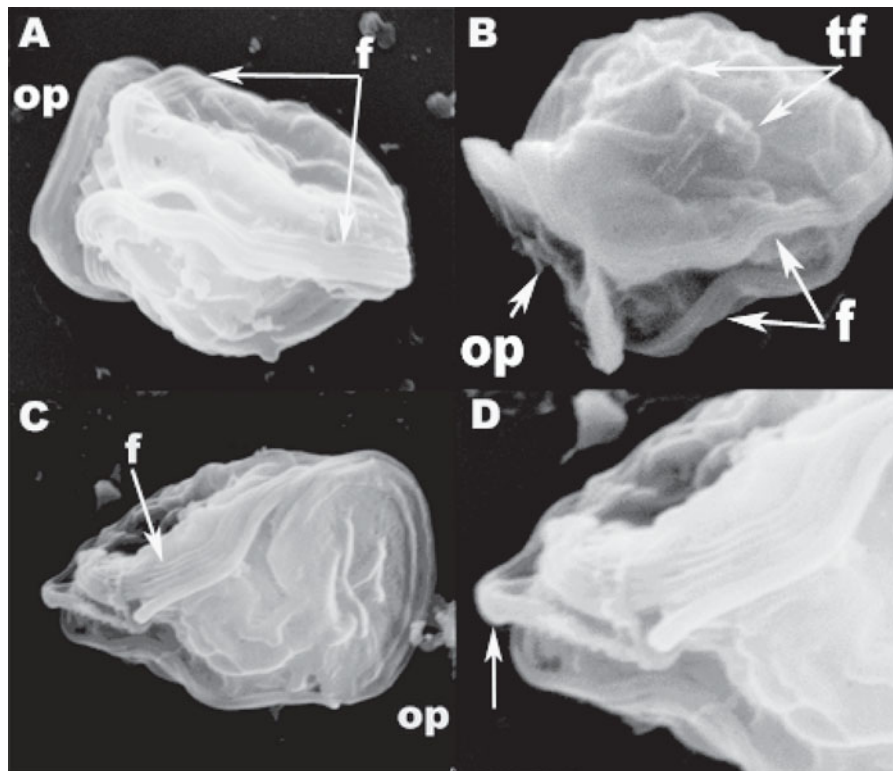


Fig. 3. Scanning electron micrographs indicating the spore ornamentation of *Haplosporidium hinei* n. sp. infecting *Pinctada maxima* in supposed order of development. (A) Spore showing a filament (f) wrapping each side of the spore to the operculum (op). (B) Spore showing the tubular filaments (tf) and spore wall filaments (f). (C) Spore showing the origin of the filaments (f) at the posterior knob-like thickening (large arrow). (D) Higher magnification view of the basal section of the spore with the posterior knob-like thickening (large arrow).

Round or elongated haplosporosomes were present that varied in diameter from 90 nm to 500 nm (mean = 256 nm; Fig. 2). Many of the haplosporosomes contained an internal membrane that was either circular or shaped as an axe head (Fig. 2). In the apical zone of the endosporoplasm, a spherule was formed by several vesicles approximately 0.1  $\mu$ m in diameter (Fig. 2). Bundles of microfilaments were also occasionally observed in the spore endosporoplasm (data not shown).

Obvious by both TEM and SEM were 2 filaments that were wound around the spore which appeared to originate from 2 posterior spore wall thickenings that resembled 'knobs' (Figs 2 and 3). The thickenings seemed to appear late in the development of the spore and were approximately 700 nm long. Both filaments passed up one side of the spore together until just below the operculum whereupon the filaments split and passed obliquely under each side of the lip of the operculum lid (Fig. 3). There was 1 filament on each side of the spore (Fig. 3). Both filaments wrapped around the spore 4 or 5 times and decreased in diameter toward the distal end (Fig. 2). The number and arrangement of the filaments was confirmed by TEM. Each filament was approximately 27  $\mu$ m in length. The filaments were derived from the spore wall and were not projections of the

epispore cytoplasm. A second set of branching tubular filaments composed of a different material was observed on the spore body although not on mature spores possessing a 'knob-like' posterior thickening (Fig. 2).

#### Taxonomic summary

*Haplosporidium hinei* Bearham, Spiers, Raidal, Jones, Burrenson and Nicholls n. sp.

#### Description and identifying characteristics

Two spore wall-derived filaments that are wound around the spore and originate from a posterior spore wall thickening. Both filaments pass up one side of the spore together until just below the operculum whereupon the filaments split and pass obliquely under each side of the lip of the opercula lid. There is 1 filament on each side of the spore. Both filaments are wrapped around the spore 4 or 5 times and decrease in diameter toward the distal end. Each filament is approximately 27  $\mu$ m in length.

#### Type host

*Pinctada maxima* (Mollusca, Bivalva, Pteriidae).

*Site of infection*

Digestive gland epithelium, gills and mantle.

*Type locality*

Carnarvon (latitude: 24°53'S, longitude: 113°40'E), Western Australia.

*Material deposited:*

H&E slides at the Western Australian Museum. Registration number WAM 227550.

*Etymology*

The epithet refers to Dr Mike Hine who has contributed a significant amount to our understanding of the haplosporidians and who originally observed this parasite.

## DISCUSSION

The parasite described in this study shows the typical morphology, spore structure and spore ornamentation of a haplosporidian species. The internal organization of the spore endosporoplasm shows a similar arrangement to other haplosporidian species. This was characterized by the presence of a basal or equatorial nucleus, an apical spherule and several electron-dense membrane-bound haplosporosomes. The presence of an orifice that is covered with an operculum suggests the parasite belongs to either *Minchinia*, *Haplosporidium* or *Bonamia*.

If the criteria proposed by Ormieres (1980) for distinguishing the genera *Haplosporidium* and *Minchinia* are followed then *Haplosporidium hinei* n.sp. was correctly assigned to *Haplosporidium* by Hine and Thorne (1998) because the filaments are derived from the spore wall and not from epispore cytoplasm. The similarity of *H. hinei* to *H. lusitanicum*, *H. pickfordi*, *H. montforti*, *H. parisi* and *H. comatulae* in the origin of the ornamentation from the basal position on the spore justifies its placement within *Haplosporidium*. The alternative genus with ornamentation originating from the spore wall is *Bonamia*. The sole member of the *Bonamia* that has been found to possess spores, *Bonamia perspora* has strap-like projections which do not originate from a basal position on the spore (Carnegie *et al.* 2006).

When compared to the study of Hine and Thorne (1998), the fixed spores in the current study, at 3.5 µm–4 µm × 2.5 µm–3.0 µm are smaller than those described previously, and Hine and Thorne (1998) also did not observe posterior knobs or surface filaments in their TEM study. Otherwise, the microfilaments and haplosporosomes observed here are consistent with those described previously.

The ornamentation on the spores of the pearl oyster parasite described by the number of filaments, their length, insertion points on the wall and organization of the filaments is unique among described haplosporidian species where spore ornamentation is known. *Haplosporidium armoricanum* from *Ostrea edulis* in Europe has paired filaments arising from each end of the spore (Azevedo *et al.* 1999). *Haplosporidium nelsoni* and *H. costale* from *Crassostrea virginica* along the east coast of the United States do not have paired filaments arising from the posterior end of the spore; rather they have wrappings around the spore (Burreson and Reece, 2006). *Haplosporidium edule* from cockles in Europe has many knobbed extensions of the spore wall, not long filaments (Azevedo *et al.* 2003).

The basal origin of the filaments described in this study is similar to that of the spores occurring in the gastropod haplosporidians of *Haplosporidium montforti*, *H. pickfordi* and *H. lusitanicum*. These species have long, spore wall-derived filaments that originate at the basal end of the spore. However, these parasites lacked the distinctive axe-shaped internal membranes observed within the haplosporosomes of the pearl oyster parasite. In addition, *Haplosporidium montforti* described infecting the abalone *Haliotis tuberculata* in Spain, has 4 filaments, 20–28 µm long, with 2 attached opposite each other at the basal end and 2 other opposing filaments attached at the apical end of the spore (Azevedo *et al.* 2006). Also, the filaments of the pearl oyster parasite are round in transverse section rather than the L, T or X-like sections of *H. montforti* (Azevedo *et al.* 2006). *Haplosporidium pickfordi* from fresh water snails in the great lakes region of the United States, has 2 posterior knob-like thickenings like the pearl oyster parasite but has approximately 9 filaments wound around the spore rather than the 2 filaments observed here (Burreson, 2001). While *H. lusitanicum* described from the European gastropod *Helcion pellucidus*, has 2 basal opposing filaments ~112 µm long, and coiled around the spore 10–13 times which are elliptical in transverse section (Azevedo, 1984).

The basal origin, arrangement of the spore wall filaments and the surface microfilaments is most reminiscent of *Haplosporidium parisi* among known species. In *H. parisi* filaments are also wound around the spore and pass obliquely under the operculum. The filaments in *H. parisi* are also circular in transverse section (unlike *H. lusitanicum*). Ormieres (1980) also described the presence of small bumps on the spore wall of *H. parisi* which appear to be similar to the microfilaments described in this study except the 'bumps' in *H. hinei* are closer together and are more focally distributed compared to *H. parisi*. *Haplosporidium hinei* n. sp. differs from *H. parisi* in the length of filaments and the presence of branching tubular filaments on the surface of the spores

(Ormieres, 1980). Filaments in *H. parisi* were up to 300  $\mu\text{m}$  long and were wrapped around the spore approximately 18 times while in the pearl oyster parasite the filaments were wrapped around the spore only 4 or 5 times and are considerably shorter at approximately 27  $\mu\text{m}$ .

A similar situation occurs with *Haplosporidium comatulae* an endoparasite of the crinoid echinoderm *Oligometra serripinna* in North Eastern Australia which also possesses spore wall filaments with a similar arrangement to *H. parisi* and *H. hinei*. Filaments in *H. comatulae* are round in cross-section but may be longer than *H. parisi* since they wrap the spore approximately 32 times (La Haye *et al.* 1984). La Haye *et al.* (1984) did not observe any internal structures within the haplosporosomes of *H. comatulae* or microfilaments on the spore wall.

The definition of *Haplosporidium* has been confounded by a lack of knowledge of the spore ornamentation of the type species *Haplosporidium scolopli* Caullery and Mesnil. Like *H. scolopli*, *H. parisi* is a parasite of serpulid polychaetes of the French Atlantic coast. Ormieres (1980) concluded *H. parisi* to be very close to *H. scolopli* and consequently, the pearl oyster parasite may also be morphologically similar to *H. scolopli*.

Speculatively, the function of the spore wall filaments may be to aid in floatation. The filaments may be extended once the spore is released to the environment. Characterization of spore ornamentation requires both TEM and SEM examination. This has not been achieved for many haplosporidian species and it will probably require DNA sequence analyses of a large number of species in the phylum to elucidate the relationships among the different morphological types. Further study is required to elucidate the relationship between the parasite described here and the haplosporidian species infecting rock oysters on the same coast. The rock oyster parasite described by Hine and Thorne (2002) also possessed a posterior thickening with associated filaments in <2% of spores.

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