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# A MOLECULAR PHYLOGENY OF THE DOVE GENUS *ZENAIDA*: MITOCHONDRIAL AND NUCLEAR DNA SEQUENCES[1]

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*Abstract.* We reconstructed a phylogeny for the seven species of doves in the genus *Zenaida* on the basis of a combined analysis of mitochondrial (ND2 and cytochrome *b*) and nuclear (fibrinogen intron 7) DNA sequences. This phylogeny, which is completely resolved, is well supported with all nodes showing greater than 50% bootstrap support. There was no significant conflict between trees based on each gene independently, although trees produced from fibrinogen intron 7 did not resolve relationships among five of the *Zenaida* species. The species status of *Z. graysoni,* as well as that of *Z. meloda,* is suggested based on their divergence from sister taxa (about 1% and 4%, respectively) and other differences. *Zenaida* can be divided into two major groups: *Zenaida asiatica* and *Z. meloda* versus *Z. aurita, Z. galapagoensis, Z. auriculata, Z. graysoni,* and *Z. macroura.*

*Key words:* *β-fibrinogen intron 7, Columbidae, cytochrome* b, *doves, molecular systematics, ND2,* Zenaida.

## INTRODUCTION

Members of the New World dove genus *Zenaida* vary considerably in the sizes of their geographic ranges. The Mourning Dove (*Zenaida macroura*) and Eared Dove (*Z. auriculata*) are distributed over North and South America, respectively, whereas the Galapagos Dove (*Z. galapagoensis*) and Socorro Dove (*Z. graysoni*) are restricted to small islands. The three remaining species have intermediate range sizes: the White-winged Dove (*Z. asiatica*) in southern North and Central America, Pacific Dove (*Z. meloda*) along the west coast of South America, and the Zenaida Dove (*Z. aurita*) in the Caribbean. These biogeographic patterns potentially make *Zenaida* a good group for exploring the relationship of relative population size and biogeography to genetic variation across species. Here we develop a phylogenetic framework for relationships among *Zenaida* species.

Monophyly of *Zenaida* is generally accepted on the basis of traditional morphological and behavioral characters (Goodwin 1983, Baptista 1997), as well as molecular data (Johnson and Clayton 2000). Based on molecular evidence, *Zenaida* is most closely related to New World doves in the genera *Leptotila* and *Geotrygon* (Johnson and Clayton 2000; Fig. 1). Understanding the evolution of biogeographic patterns in *Zenaida* requires a well-supported phylogeny for the genus. We used sequences of the mitochondrial genes cytochrome *b* (cyt *b*) and NADH dehydrogenase subunit 2 (ND2), as well as the nuclear gene β-fibrinogen intron 7 (FIB7), to reconstruct a phylogeny for *Zenaida.* We compare the relative utility of mitochondrial and nuclear genes for reconstructing a phylogeny of this genus. Although the monophyly of *Zenaida* is not controversial, the species status of *Z. graysoni* and *Z. meloda* remains uncertain (Goodwin 1983, Baptista 1997). We assessed the status of these and other *Zenaida* species on the basis of genetic divergence from sister taxa, relative to the divergences between samples of the same species.

## METHODS

### SAMPLES

We obtained muscle tissue or feathers (from live birds) of *Zenaida, Leptotila,* and *Columba* species from the sources indicated in Table 1. Johnson and Clayton (2000) identified *Leptotila* as the sister group of *Zenaida,* and *Columba* as a more distant relative of the *Zenaida–Leptotila* clade (Fig. 1).

### DNA SEQUENCING

We extracted total genomic DNA from all samples using a Qiagen tissue extraction kit (Qiagen, Valencia, California). We followed manufacturer's protocols except in the case of feather

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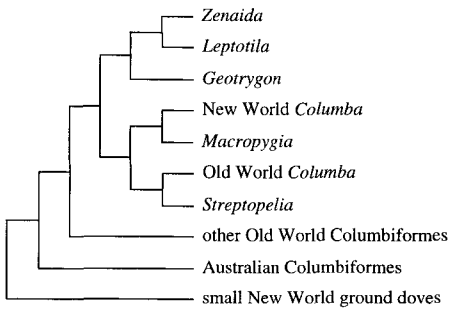


FIGURE 1. Phylogeny from DNA sequences for Columbiformes (Johnson and Clayton 2000) indicating the position of *Zenaida* among major lineages of Columbiformes.

samples, where we added 30 μl of 10% DTT to the digestion mix. We next amplified the mitochondrial cytochrome *b* using the primers L14841 (Kocher et al. 1989) and H4a (Harshman 1996). The primers L5215 (Hackett 1996) and H6313 (Johnson and Sorenson 1998) were used to amplify ND2, and the primers FIB-BI7U and FIB-BI7L (Prychitko and Moore 1997) were used for FIB7. Because nuclear copies of mitochondrial genes can potentially confound avian mitochondrial sequences, we took several precautions against nuclear copies as suggested by Sorenson and Quinn (1998). We avoided blood samples which have a relatively high copy number of nuclear genes compared to mitochondrial genes. We amplified mitochondrial genes in large (ca. 1,100 bp) pieces and separately from each other. We also checked sequence chromatograms for ambiguities and the sequences for stop codons and insertions or deletions. We found no evidence for nuclear copies of mitochondrial genes in our sequences.

PCR amplifications (50 μl total) included 1 μl Thermo flavus polymerase (Epicentre Technologies, Madison, Wisconsin), 3 μl of a 10 μM solution of each primer, 3.9 μl of $MgCl_2$, 2.5 μl of 20X reaction buffer, 35 μl distilled water, and between 1 and 4 μl of each DNA extract. We performed all amplifications using a Perkin Elmer 9700 Thermocycler with a 2-min 93°C melt followed by 35 cycles of 30 sec at 93°C, 30 sec at 46°C, and 30 sec at 72°C. This was followed by a 7-min 72°C extension and a 4°C hold. We purified amplification products using a Qiagen PCR purification kit and performed sequencing reactions using a BigDye Terminator kit (Perkin Elmer, Norwalk, Connecticut). The sequencing reactions included either the amplification primers or the additional internal primers: H15299 (Kocher et al. 1989) and L15517 (Johnson and Sorenson 1998) for cyt *b*; L5758 (Johnson and Sorenson 1998) for ND2; and FIB-DOVEF and FIB-DOVER (Johnson and Clayton 2000) for FIB7. Samples were electrophoresed on an ABI 377 automated sequencing apparatus. We aligned and reconciled the chromatograms from complementary strands using Sequencher 3.1 (GeneCodes, Madison, Wisconsin). We also aligned sequences between species with Sequencher. This produced sequences that included a large portion of cyt *b* (1,070 bp), complete ND2 (1,092 bp), and complete FIB7 (ca. 1,100 bp) genes and some flanking regions for each species (GenBank

TABLE 1. Specimens used in the phylogenetic analysis.

| Species | Common name | Source[a] |
|---|---|---|
| *Zenaida macroura* | Mourning Dove | KPJ97-005, Arizona |
| *Zenaida macroura* | Mourning Dove | DHC98-004, Texas |
| *Zenaida graysoni* | Socorro Dove | LSUMNS, B-23847[b] |
| *Zenaida auriculata* | Eared Dove | FMNH, PS-003, Argentina |
| *Zenaida galapagoensis* | Galapagos Dove | Tracy Av., UT KPJ97-006[b] |
| *Zenaida galapagoensis* | Galapagos Dove | KPJ98-002, Galapagos |
| *Zenaida aurita* | Zenaida Dove | FMNH, SML87-057, Jamaica |
| *Zenaida aurita* | Zenaida Dove | FMNH, SML87-062, Jamaica |
| *Zenaida asiatica* | White-winged Dove | KPJ97-002, Arizona |
| *Zenaida asiatica* | White-winged Dove | DHC98-001, Texas |
| *Zenaida meloda* | Pacific Dove | LSUMNS, B-5236, Peru |
| *Leptotila rufaxilla* | Grey-fronted Dove | KUMNH, B793, Peru |
| *Columba plumbea* | Plumbeous Pigeon | FMNH, ATP86-136, Brazil |

[a] KPJ = first author, DHC = second author, LSUMNS = Louisiana State University Museum of Natural Science, FMNH = Field Museum of Natural History, KUMNH = Kansas Museum of Natural History.
[b] Captive individuals.

accession numbers AF182658, AF182665–AF182671, AF182691, AF182698–AF182704, AF251530–AF251547, AF254349–AF254350).

### PHYLOGENETIC ANALYSIS

We constructed trees using unordered parsimony for all genes independently, and combined, using PAUP* (Swofford 1999) with branch and bound searches. Trees were rooted on *Columba plumbea,* the Plumbeous Pigeon. We evaluated support for all trees using bootstrap data resampling with 1,000 full-heuristic replicates (Felsenstein 1985). We compared the phylogenetic information in all gene regions using the partition homogeneity test (Farris et al. 1994, 1995, Swofford 1999). We compared rates of divergence between genes by plotting percent sequence divergence in cyt *b,* ND2, and FIB7 against each other. To explore the sensitivity of the phylogeny to weighting scheme, we conducted parsimony searches using 1:1, 2:1, and 5:1 weighting of transversions over transitions. To explore the sensitivity of the phylogeny to method of analysis, we also performed a maximum likelihood analysis in PAUP*. We used the simplest model that could not be rejected in favor of a more complex model using the likelihood-ratio test procedure outlined by Huelsenbeck and Crandall (1997). This procedure resulted in a model with empirically estimated base frequencies, general time reversible substitutions, and rate heterogeneity according to a gamma distribution. We used the quartet puzzling algorithm (Strimmer and von Haeseler 1996) as implemented in PAUP* to derive reliability scores for each branch to obtain a measure of support for branches in the maximum likelihood tree. We used the maximum likelihood model on the mitochondrial sequences to test whether a molecular clock could be rejected for these sequences using a likelihood ratio test. We also tested whether the phylogenetic hypothesis of Goodwin (1983), who unites *Z. aurita* and *Z. galapagoensis* because they have 12 tail feathers instead of 14, could be rejected using a Templeton (1983) test.

### BIOGEOGRAPHY

To attempt to reconstruct ancestral areas of origin, we used both Brooks' (1990) method of unordered parsimony and Ronquist's (1997) dispersal-vicariance analysis. We used MacClade (Maddison and Maddison 1992) to reconstruct ancestral areas under Brooks' (1990) method. We used DIVA (Ronquist 1996) to perform the dispersal-vicariance analysis. Brooks' method generally favors dispersal over vicariance, whereas Ronquist's method tends to favor vicariance as an explanation for biogeographic patterns.

## RESULTS

For cyt *b,* 271 of 1,070 (25.3%) positions were variable and 172 (16.1%) of these were potentially informative. ND2 showed variation at 337 of 1,092 (30.9%) positions, and 222 of these (20.3%) were informative. FIB7 was much less variable, 93 of 1,119 positions (8.3%), and contained few informative positions, 27 (2.4%). Rates of substitution were relatively similar for cyt *b* and ND2, and both were substantially higher (ca. five times) than the rate for fibrinogen intron 7 (Fig. 2). This rate is estimated from the initial slope of the plot of pairwise divergences in the mitochondrial genes versus the nuclear intron. Rate differences between cyt *b* and ND2 also are apparent. Although cyt *b* and ND2 appear to accumulate substitutions at nearly the same rate at low divergences (less than 10%, Fig. 2a), ND2 appears to accumulate more substitutions than cyt *b* at high divergences (greater than 10%). This result probably is due to a higher level of multiple substitutions in cyt *b*; note that divergence in cyt *b* appears to level off when compared to divergence in FIB7 (Fig. 2b), whereas divergence in ND2 does not (Fig. 2c).

Unweighted parsimony trees based on the three genes individually differed slightly (not shown). Nodes that were in common between all three trees had relatively high bootstrap support (>74% in all trees), whereas nodes that differed between the three trees were supported in less than 65% of bootstrap replicates in any one tree. The phylogeny based on cyt *b* showed the most resolution, whereas the phylogeny based on FIB7 could not resolve relationships among *Z. aurita, Z. galapagoensis, Z. auriculata, Z. macroura,* and *Z. graysoni.* A partition homogeneity test indicated that the DNA sequence data from three genes do not differ significantly ($P = 0.42$), so we combined the gene regions in further analyses. Because the FIB7 gene has a much slower rate of substitution and sorts independently from the mitochondrial genome, we also constructed trees based only on the mitochondrial gene regions. The parsimony trees

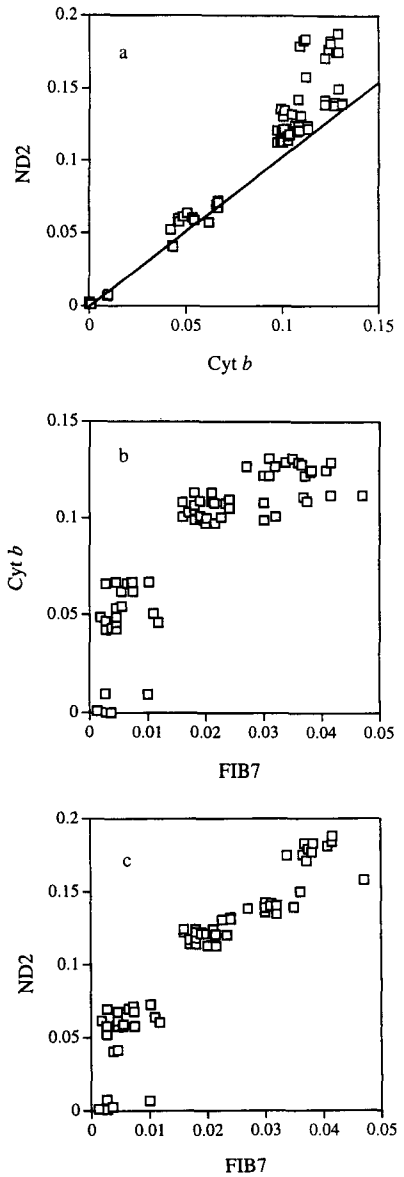


FIGURE 2. Percent sequence divergences from pairwise comparisons in *Zenaida* and outgroups. (a) ND2 versus cyt *b*; the line indicates expectation given equal rates of substitution, (b) cyt *b* versus FIB7, (c) ND2 versus FIB7.

based on all genes combined or on just the mitochondrial genes were identical (Fig. 3). This tree was completely resolved and did not change with transversion weighting; thus, choice of transversion weighting scheme does not impact the results. All nodes are supported at over 50% in bootstrap replicates. Maximum likelihood

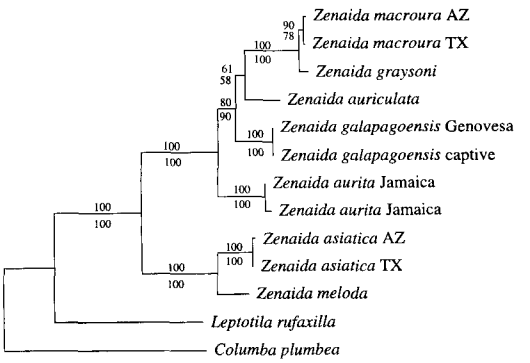


FIGURE 3. Tree derived from unordered parsimony of combined cyt *b*, ND2, and FIB7 DNA sequences (length = 984, RC = 0.635) for *Zenaida* and outgroups. Numbers above branches indicate percent support from 1,000 full heuristic bootstrap replicates. Numbers below branches are reliability scores from the maximum likelihood quartet puzzling analysis. Branch lengths are proportional to reconstructed DNA sequence changes. The topology shown also was recovered in 2:1 and 5:1 transversion weighted parsimony searches.

analysis yielded a tree identical to the combined parsimony tree. Quartet puzzling reliability scores were greater than 50% for all nodes in the tree. *Zenaida* appears as a monophyletic genus, with the limited number of outgroups in the current study. The monophyly of *Zenaida* also was supported in an analysis of many additional outgroups (Johnson and Clayton 2000). Monophyly of all species sampled by more than one individual was recovered in the combined tree: *Z. asiatica, Z. macroura, Z. galapagoensis,* and *Z. aurita* (Fig. 3). Using the Templeton (1983) test, we could reject Goodwin's (1983) phylogenetic hypothesis for *Zenaida* (Fig. 4): $P = 0.018$.

For the two mitochondrial genes, the divergence between *Zenaida asiatica* and *Zenaida meloda* is 4.2%. The divergence between *Z.*

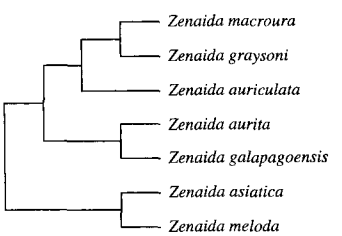


FIGURE 4. Phylogenetic hypothesis for *Zenaida* suggested by Goodwin (1983).

*graysoni* and *Z. macroura* is 0.9%. These values are much higher (ca. 40 and 10 times, respectively) than the divergence between individuals from different localities or recognized subspecies within *Zenaida* species: 0.09% for *Z. asiatica,* 0.1% for *Z. macroura,* 0.05% for *Z. galapagoensis,* and 0.05% for *Z. aurita.* The molecular clock constraint could not be rejected for the mitochondrial sequences under the maximum likelihood model ($P > 0.10$).

Analysis of ancestral areas by Brooks' (1990) method indicated an origin of Central America and the Caribbean or South America for *Zenaida.* Dispersal-vicariance analysis (Ronquist 1997) provides an equivocal reconstruction of the ancestral area for *Zenaida* and tends to reconstruct ancestral occurrence in several areas.

## DISCUSSION

A phylogeny based on over 3,000 base pairs of sequence including both mitochondrial (cyt *b* and ND2) and nuclear (FIB7) genes was well resolved for the genus *Zenaida.* All nodes received >50% bootstrap support, and tree topology did not change with transversion weighting or between parsimony and maximum likelihood. Substitution rates and the potential for multiple substitution can influence the ability of genes to recover phylogeny. Comparisons of the substitution rate of cyt *b* and ND2 indicated a similar rate of substitution at low divergences (< 10% cyt *b* divergence, see Fig. 2a). At greater than 10% cyt *b* divergence, ND2 appears to accumulate recovered substitutions at a higher rate, i.e., ND2 is less subject to multiple substitution. Thus, cyt *b* and ND2 may be equally useful for phylogeny reconstruction at divergences less than 10%, but ND2 may perform better at higher divergences. This difference in pattern of multiple substitution between cyt *b* and ND2 may not be the same in all avian groups. For example, a lower rate of multiple substitution in ND2 also was found in comparisons of these two genes for tanagers (Hackett 1996), orioles (Omland et al. 1999), blackbirds (Johnson and Lanyon 1999), and cuckoos (Johnson et al. 2000). However, in dabbling ducks, cyt *b* and ND2 show a similar rate of substitution up to at least 14% cyt *b* sequence divergence (Johnson and Sorenson 1998).

Rates of substitution in vertebrate nuclear genes are generally slower than rates in mitochondrial genes (Brown et al. 1979). Our results confirm this because the nuclear intron (FIB7) showed an approximately five times slower rate of substitution than did the two mitochondrial protein coding genes. This slow substitution rate made it difficult to resolve relationships between *Zenaida* species based on FIB7 alone. Very few differences were observed between species in FIB7, resulting in a lack of potentially phylogenetically informative sites. In addition, species monophyly within *Z. macroura* and within *Z. aurita* was not recovered using just FIB7 because of a lack of differentiation from related species. However, the strongly supported split between *Z. asiatica* + *Z. meloda* and all other species of *Zenaida* was recovered using FIB7 alone, and more importantly, the signal between the nuclear and mitochondrial genes was not significantly incongruent.

Our molecular phylogeny is the first assessment of relationships among all species of *Zenaida* using modern systematic methods. The combined phylogeny indicates that the White-winged Dove clade (*Z. asiatica* + *Z. meloda*) is sister to all other *Zenaida.* This relationship is consistent with previous morphological and behavioral assessments of *Zenaida* relationships (e.g., Fig. 4). The relationships among the other species of *Zenaida* indicate that *Z. aurita* is sister to the remaining *Zenaida* species. The relationships among *Z. auriculata, Z. galapagoensis,* and *Z. macroura* + *Z. graysoni* are not well supported, but both the parsimony and maximum likelihood trees indicate that *Z. galapagoensis* is sister to all the other species in this group (Fig. 3). In contrast, Goodwin (1983) placed *Z. aurita* and *Z. galapagoensis* as sisters and *Z. macroura* and *Z. auriculata* as sisters, because *Z. aurita* and *Z. galapagoensis* both have 12 tail feathers instead of the 14 typical of other *Zenaida* (Fig. 4). Our phylogenetic tree implies that either 12 tail feathers is independently derived in *Z. aurita* and *Z. galapagoensis* or is derived once and then lost in the *Z. auriculata, Z. macroura, Z. graysoni* clade.

Data from genetic divergences can provide information on species limits and on the timescales of diversification. The level of mitochondrial genetic divergence between the Central and North American *Z. asiatica* and the South American *Z. meloda* (4.2%) is high in relation to a mere 0.09% divergence between White-winged Doves from Arizona and Texas. The split between *Z. asiatica* and *Z. meloda* also is recov-

ered using the slowly evolving nuclear gene. These high divergences would generally only accumulate under long periods of no gene flow between populations and indicate that *Z. meloda* should be recognized as a separate species from *Z. asiatica.* Although the plumage of these two species is quite similar, *Z. asiatica* and *Z. meloda* differ in soft-part coloration and in vocalizations, further supporting their species status (Baptista 1997). The extent of reproductive isolation between these two species is unknown, but under phylogenetic (Cracraft 1983, McKitrick and Zink 1988) or evolutionary (Simpson 1951) species concepts their recognition is supported.

We could not reject a molecular clock constraint under the maximum likelihood model for the mitochondrial genes ($P > 0.10$). The mitochondrial divergence between Mourning (*Z. macroura*) and Socorro (*Z. graysoni*) Doves is 0.9%, suggesting that the Socorro Dove has been evolving independently from the Mourning Dove for approximately 450,000 years (assuming a mitochondrial molecular clock of 2% per million years, Klicka and Zink 1997). The Socorro Dove also differs from the Mourning Dove in plumage, vocalizations, and visual displays (Baptista et al. 1983, Baptista 1997). Hybrids between the two species are in many cases sterile (Baptista 1997). Given these differences and our inferred lack of continued gene flow, we confirm that the Socorro Dove, now extinct in the wild (Jehl and Parkes 1982), should likely be recognized as a species separate from the Mourning Dove under any species concept.

In contrast to the high divergence between Socorro Dove and Mourning Dove, divergence within the Mourning Dove in North America appears to be low. Using mitochondrial restriction sites, Ball and Avise (1992) reported a maximum mitochondrial divergence of approximately 0.5% among Mourning Dove haplotypes. They also found the same haplotypes in both eastern and western subspecies, resulting in a lack of monophyly for these subspecies. Consistent with these results, we found a low mitochondrial divergence (0.1%) between the eastern and western individuals that we sampled.

## BIOGEOGRAPHY

Biogeographic reconstruction under vicariance scenarios (Ronquist 1997) provide little resolution on the ancestral range of *Zenaida.* Analysis under Brooks' (1990) unordered parsimony method suggest a South American or Central American origin. The basal species in the *Zenaida* tree occur in South and Central America/Caribbean, resulting in this conclusion. Representatives of the closely related genera *Leptotila* and *Geotrygon* are also distributed in both these areas, so including many representatives of these genera in an analysis will be necessary to resolve *Zenaida* origins in more detail.

Assuming that colonization of islands from continental areas is much more likely than the reverse, the Socorro Dove is probably derived from the Mourning Dove as has been suggested by earlier authors (Goodwin 1983, Baptista 1997). The estimated age of the colonization of Socorro Island from a molecular clock assumption is 450,000 years ago, which is close to the approximate geological age of the island of 500,000 years (L. Baptista, pers. comm.). The colonization of the Galapagos by *Zenaida* predates the split between Mourning and Eared Doves (slightly over 2 million years ago), but appears to have occurred a considerable amount of time after the geologic formation of the Galapagos. Another interesting pattern of divergence is the relatively similar divergence between North and South American counterparts: Mourning vs. Eared Doves and White-winged vs. Pacific Doves. We estimate the speciation events between these counterparts to be between 2 million and 2.7 million years ago. This is after the formation of the Central American land bridge (ca. 3 million years ago) and suggests that perhaps the formation of the bridge facilitated dispersal of *Zenaida* into Central and North America leading to speciation. In general it appears that dispersal rather than vicariance has been an important cause of speciation in *Zenaida.*

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