Analysis of Mitochondrial DNA Variability and Genetic Structure in Populations of New World Screwworm Flies (Diptera: Calliphoridae) from Uruguay

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ABSTRACT The New Word screwworm, *Cochliomyia hominivorax* (Coquerel 1858) (Diptera: Calliphoridae), is one of the most important insect pests of livestock in the Neotropical region. In this work, polymerase chain reaction-restriction fragment length polymorphism of mitochondrial DNA (mtDNA) was used to study the diversity and population structure of seven geographically distinct populations of *C. hominivorax* from most of the important livestock areas in Uruguay. The control region (A+T/12S) and subunits 1 and 2 of cytochrome oxidase (*cox*1/*cox*2) were amplified and digested with restriction endonucleases. Nine haplotypes were observed among the populations sampled. The mean nucleotide diversity and the haplotype diversity indicated high mtDNA variability in this species. The similarity index, average nucleotide divergence, and analysis of molecular variance results showed no evidence of subpopulation differentiation, indicating that the *C. hominivorax* populations of Uruguay form a single panmitic population. The distribution pattern of the genetic variation in natural populations of *C. hominivorax* and the implications of these results for establishing control program are discussed.

KEY WORDS screwworm, mitochondrial DNA, polymerase chain reaction-restriction fragment length polymorphism, population structure, Uruguay

THE NEW WORLD SCREWWORM, *Cochliomyia hominivorax* (Coquerel 1858) (Diptera: Calliphoridae), is an obligate ectoparasite that causes myiasis in warm-blooded vertebrates throughout the Neotropical region (Guimarães et al. 1983). Adult females lay eggs in open wounds where the emerging larvae feed (Guimarães et al. 1983). The infestation by these larvae generates an exudate that stimulates more females to lay their eggs in the wound, resulting in injuries containing hundreds to thousands of larvae (Thomas and Mangan 1989). This mode of infestation makes *C. hominivorax* one of the most important pests of livestock and one of the most expensive to control and treat (Hall and Wall 1995).

Historically, the distribution of *C. hominivorax* extended from the southern United States to Argentina. However, this species has been successfully eradicated from North and most of Central America by using the sterile insect technique (SIT), but it still occurs in the Caribbean islands and South America, except for Chile (Wyss and Galvin 1996, IAEA/FAO 2000). In 1988, *C. hominivorax* was recorded outside of the Americas for the first time (in Libya), but it was efficiently combated using SIT (Vargas-Teran et al. 1994).

In Uruguay, as in other South American countries, *C. hominivorax* is one of the most important insect pests and represents a significant health problem for the livestock industry, often causing great economic losses (Carballo et al. 1990, IAEA/FAO 2000).

Because of the economic importance of *C. hominivorax* and its influence on the trade of live animals among infested and noninfested countries, international efforts have been aimed at designing a program to control and eventually eradicate this species from endemic areas and to prevent invasions into screwworm-free areas. A fundamental component of a successful pest management strategy, such as SIT, is a good understanding of the genetic diversity and struc-

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ture of the target populations (Roehrdanz 1989, Infante-Vargas and Azeredo-Espin 1995).

Mitochondrial DNA (mtDNA) is a suitable marker for studying microevolutionary processes in animal populations and can be a suitable marker for estimating the genetic variability within populations (Avise et al. 1987, Avise 1994, Rokas et al. 2003). The restriction fragment length polymorphism (RFLP) analysis of mtDNA has been successfully used to study the genetic variability in *C. hominivorax* populations and has revealed high variation in this molecule (Roehrdanz and Johnson 1988, Roehrdanz 1989, Infante-Vargas and Azeredo-Espin 1995, Taylor et al. 1996). Another approach for studying mtDNA variation is polymerase chain reaction (PCR)-RFLP analysis. This powerful method has been used in several population analyses (Ross et al. 1997, Dueñas et al. 2002).

The aim of this study was to examine the genetic variability among geographically distinct populations of *C. hominivorax* from Uruguay, at the southern limit of this species' distribution, by using mtDNA PCR-RFLP analysis. Inferences regarding the degree of isolation of the populations analyzed were made by testing the subdivided population hypothesis by using analysis of molecular variance (AMOVA).

Materials and Methods

Samples. Larvae of *C. hominivorax* from seven Uruguayan localities, including important livestock areas, were obtained from wounded sheep, dogs, or cattle in January 2003. Collected larvae were reared in the laboratory for species identification (Guimarães et al. 1983) and were allowed to pupate or fixed in 100% ethanol in the third instar. The adults that emerged and the fixed larvae were used for DNA extractions. Total nucleic acids were isolated using the phenol: chloroform method, as reported in Infante-Vargas and Azeredo-Espin (1995), and the DNA extracted was stored at −20°C. In total, 175 larvae were obtained from 48 wounds.

The geographic locations of the seven areas sampled (all low hills and plains in the transition from the Argentinean pampas to the hilly uplands of southern Brazil) are Bañados de Medina-Cerro Largo (32° 23′ 00 S, 54° 21′ 00 W), Cerro Colorado-Florida (33° 52′ 00 S, 55° 33′ 00 W), Colonia-Colonia (34° 20′ 00 S, 57° 86′ 67 W), Dayman-Paysandú (31° 33′ 00 S, 57° 57′ 00 W), Joaquín Suárez-Canelones (34° 44′ 01 S, 56° 02′ 12 W), Paso Muñoz-Salto (31° 27′ 00 S, 56° 23′ 00 W) and San Antonio-Salto (31° 24′ 00 S, 57° 58′ 00 W) (Fig. 1).

For haplotype frequency estimations and genetic comparisons, each haplotype found in a wound was considered only once. This approach avoids a bias in the analysis caused by sampling the same mitochondria, but it could bias the results toward higher estimates of diversity, because common haplotypes would be counted only once in multiple infections. However, this conservative approach that reduces sample size was preferred to an artificial overestimation of the precision of this study. Moreover, because sibling larvae have a gregarious behavior, this putative bias tends to be actually minimized.

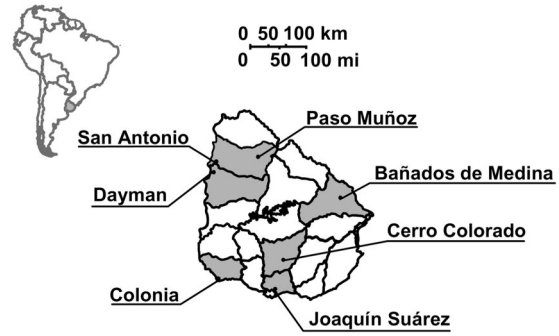

Fig. 1. Geographic locations of the screwworm populations sampled in this study.

Using this approach, the number of *C. hominivorax* considered in the analysis of genetic variation was 65, and it was sufficient to provide information at the hierarchical level of the population.

Amplifications. Two specific mtDNA regions were amplified: one region with 2100 bp included the complete control region and partial rRNA 12S sequences (A+T-rich/12S) and another region with 2360 bp included the entire sequences of cytochrome oxidase subunits 1 and 2 (*cox*1/*cox*2). PCR assays were done as described by Lessinger and Azeredo-Espin (2000) and Litjens et al. (2001), by using a PTC-200 (MJ Research, Watertown, MA) thermal cycler. The UBC-insect mtDNA oligonucleotide set described by Simon et al. (1994) was used for A+T rich/12S amplification with the primers TM-N-193 and SR-J-14233; the primers TY-J-1460 and TK-N-3785 were used for *cox*1/*cox*2 amplification. The PCR products were evaluated by electrophoresis in 1.0% agarose gels, stained with ethidium bromide (EtBr), in 1× TAE (40 mM Tris-acetate, 1 mM EDTA) buffer.

RFLP Procedures. For a preliminary survey to detect mtDNA polymorphisms, three individuals were randomly chosen from different Uruguayan and Brazilian localities. The Brazilian samples were from previous DNA extractions used in RFLP analysis of mtDNA (Infante-Vargas and Azeredo-Espin 1995). The samples were amplified and digested to provide initial information on the restriction sites present in the mtDNA regions analyzed and their usefulness for population analysis.

Aliquots of PCR products of the A+T-rich/12S and *cox*1/*cox*2 regions were single digested for 4 h at 37°C, according to the enzyme supplier's protocols (Invitrogen, Carlsbad, CA, and Pfizer, Inc., New York, NY). The digested fragments were separated by electrophoresis in 2% agarose gels, stained with EtBr, and photographed using the Kodak EDAS 290 software in an UV *trans*-illuminator. The size of the fragments was estimated by comparison with the molecular size standard DNA Ladder Plus 1Kb (12 kb-100 pb, Invitrogen) by using regression analysis, carried out manually. Digestions with enzymes that produced different re-

Table 1. Diagnostic restriction patterns obtained by PCR-RFLP of *C. hominivorax* from Uruguay

Region	Enzyme	mtDNA	Pattern (bp)			
A+T/12S	*Dra* I	A	800	320	200	200
		B	1000	320	200	
		C*	1900	320	200	200
*cox*1/*cox*2	*Ase* I	A	1100	460	350	220
		B	1100	680	350	
	Msp I	A	1500	480		
		B	1400	480		
		C	1500	300		
		D	870	680	300	
		E	1400	300		

Capital letters indicate polymorphic restriction patterns. Fragments <200 bp were not considered. The existence of an unknown no. of small fragments accounts for the sum of the fragments being less than that of the intact amplified segment. C* represents the fragment size polymorphism.

striction patterns were repeated to confirm that the observed patterns do not result from partial digests. Fragments <200 bp were not included in the analysis.

The PCR products of the A+T-rich/12S sequences were digested with nine restriction endonucleases: *Cla* I, *Dra* I, *Eco*R V, *Hae* III, *Hind* III, *Msp* I, *Mun* I, *Ssp* I, and *Sau*96 I; three of the enzymes (*Hae* III, *Msp* I, and *Mun* I) did not cut the PCR product, five of the enzymes (*Cla* I, *Eco*R V, *Hind* III, *Ssp* I, and *Sau*96 I) produced only a single cleavage pattern for the samples analyzed, and only *Dra* I detected polymorphism in this region (Table 1). The PCR products of the *cox*1/*cox*2 region were digested with 13 restriction endonucleases: *Ase* I, *Dde* I, *Dra* I, *Eco*R I, *Eco*R V, *Hind* III, *Hpa* I, *Msp* I, *Rsa* I, *Sma* I, *Ssp* I, *Sst* I, and *Xho* I; four of the enzymes (*Eco*R I, *Hind* III, *Hpa* I, and *Sma* I) did not cut the PCR product, seven of the enzymes (*Dde* I, *Dra* I, *Eco*R V, *Rsa* I, *Ssp* I, *Sst* I, and *Xho* I) produced only a single cleavage pattern for the samples analyzed and the restriction endonucleases *Ase* I and *Msp* I showed diagnostic restriction patterns (Table 1). The enzymes were selected based on a previous RFLP analysis and PCR-RFLP results for *C. hominivorax* (Infante-Vargas and Azeredo-Espin 1995, Litjens et al. 2001) and on an analysis of the mtDNA A+T rich/12S and *cox*1/*cox*2 sequences available for mtDNA *C. hominivorax* species (Lessinger et al. 2000) by using the WebCutter software (Heiman 1997). The Uruguayan samples were analyzed after the diagnostic enzymes had been selected for both regions.

Data Analysis. *Haplotype Analyses.* The different restriction patterns for both regions obtained with each enzyme were designated with capital letters, according to the order in which they were detected. For each individual, these letters were compiled into a composite haplotype designated by numbers (Table 2). Each haplotype was scored by a vector of 0s and 1s (absence and presence of a fragment, respectively) that represented the components of their PCR-RFLP phenotypes, and a matrix was constructed to be used as an input file in the analyses of genetic variation. The evolutionary distance (d) between all pairwise comparisons of haplotypes was estimated according to Nei and Li (1979) and Nei (1987) (equation 5.55) by using the REAP software (McElroy et al. 1992), considering the different size of restriction endonuclease recognitions sequences (r-value = 4 or 6) (Nei 1987).

Diversity. The genetic diversity within each population analyzed was interpreted using the estimate of haplotype diversity (Hs) and nucleotide diversity (π). Haplotype frequency distributions for each population and the associated d values among haplotypes were used to estimate these diversity indices. Haplotype diversity was estimated according to Nei (1987) (equations 8.4, 8.5, and 8.12), and nucleotide diversity was estimated according to Nei and Tajima (1981) by using the REAP software (McElroy et al. 1992).

Population Differentiation. The variation among the populations analyzed and the population differentiation were interpreted using different indices. The similarity index (F) or the proportion of shared fragments between populations was calculated for each possible pairwise comparison of populations. This index was estimated according to Nei and Li (1979) as $F = 2N_{xy}/(N_x + N_y)$, where N_x and N_y are the numbers of fragments in populations x and y, respectively, and N_{xy} is the number of fragments shared by the two populations. The nucleotide divergence (δ) was estimated according to Nei and Tajima (1981) by using the REAP software (McElroy et al. 1992).

Table 2. Distribution of mtDNA haplotypes in Uruguayan populations

Haplotype		JoS	BaM	Col	CeC	PaM	Day	SaA	Total	Freq
1	AAC	2 (7)	5 (14)	3 (12)	6 (15)	3 (12)	7 (17)	7 (10)	33 (87)	0.51
2	AAD		1 (3)						1 (3)	0.02
3	ABA	3 (17)	4 (11)		3 (5)	3 (8)		1 (1)	14 (42)	0.22
4	ABB		2 (8)	2 (10)	6 (10)	1 (2)		1 (4)	12 (34)	0.18
5	BAC						1 (1)		1 (1)	0.02
6	ABE				1 (2)				1 (2)	0.02
7	CAC							1 (3)	1 (3)	0.02
8	AAA						1 (2)		1 (2)	0.02
9	ABC						1 (1)		1 (1)	0.02
Total		5 (24)	12 (36)	5 (22)	16 (32)	7 (22)	10 (21)	10 (18)	65 (175)	1.00
No. wounds		5	8	5	10	4	9	7	48	

The haplotypes are designated by a number and a combination of the three restriction enzymes patterns (*Dra*I, *Ase*I, and *Msp*I). The numbers in parentheses indicate the total number of individuals found with a haplotype and those without parentheses indicate the number of individuals used for population analyses. BaM, Bañados de Medina; CeC, Cerro Colorado; Col, Colônia; Day, Dayman; JoS, Joaquín Suárez; PaM, Paso Muñoz; SaA, San Antonio; Freq, frequency.

Table 3. Estimates for the indices of genetic variation within and among populations

Genetic variation within populations		Genetic variation among populations			
π	*Hs*	*F*	δ	Φ_{ST} (pd)	Φ_{ST} (nd)
0.022975 ($\pm$ 2.09 $\times$ 10^{-5})	0.6355 ($\pm$ 1.17 $\times$ 10^{-3})	0.967	0.000555 ($\pm$ 1.2 $\times$ 10^{-6})	0.0762 (df = 6, 58; P = 0.0865)	0.1449 (df = 6, 58; P = 0.8768)

π, nucleotide diversity; *Hs*, haplotype diversity; *F*, similarity; δ, nucleotide divergence; Φ_{ST} (pd), *F*-statistic based on pairwise differences ($=\theta_W$); Φ_{ST} (nd), *F*-statistic based on Nei's evolutionary distance (d).

To study the distribution of genetic variation within and among populations, an AMOVA was done using ARLEQUIN version 2.0 (Schneider et al. 2001). The degree of isolation of the populations was interpreted using the Φ-statistic (Φ_{ST}) parameter. This analysis was done by considering the number of pairwise differences and the evolutionary distance (d) between haplotypes. The significance of the variance components and Φ_{ST} was computed using a nonparametric permutation test (Excoffier et al. 1992).

Results

Genetic Variation. Two fragments were observed after amplification of the A+T-rich/12S sequences. The most frequent fragment in the Uruguayan populations contained ≈2100 bp and occurred in 98% of the samples; this fragment was previously described by Litjens et al. (2001). The second fragment contained ≈3200 bp and was identified in three individuals from the same wound in the locality of San Antonio. The nature of this variation is still under investigation. The amplified *cox*1/*cox*2 sequences showed no size polymorphism.

Digestion of the A+T-rich/12S sequences with the enzyme *Dra*I produced three diagnostic patterns. Patterns A and B were digestion products of the 2100-bp fragment (restriction site polymorphism), and pattern C was identified when the 3200-bp fragment was digested (fragment size polymorphism) (Table 1). Pattern A occurred in 96% of the samples analyzed. For *cox*1/*cox*2 sequences, the enzyme *Msp*I yielded five diagnostic patterns for the populations, whereas the enzyme *Ase* I presented two different patterns (Table 1). The existence of an unknown number of small fragments meant that the total size for the sum of the fragments was less than that of the intact amplified fragment.

We examined ≈25% (≈4.2 kb) of the *C. hominivorax* mitochondrial genome (Lessinger et al. 2000), and nine haplotypes were identified on the basis of the restriction patterns. Table 2 shows the distribution and frequency of the haplotypes found at the different Uruguayan localities sampled. Haplotype 1 was the most frequent (51%) in the sample as a whole and at most of the localities, and it was present in all populations. Haplotypes 3 and 4 represented 22 and 18% of the total sample, respectively, and they were widely distributed among the populations. The distance estimates between pairs of common haplotypes was d(1vs3) = 0.0227, d(1vs4) = 0.0719) and d(3vs4) = 0.0110. The others six haplotypes had a local distribution and represented a very low proportion of the total sample (<2% each).

Only one of these six rare haplotypes was found in the three southern locations (Colonia, Joaquín Suárez, and Cerro Colorado) near the Uruguayan coast, and five of these haplotypes were found near the border with Brazil in northwestern and northeastern locations. Although this distribution of haplotype would suggest a decrease in diversity at the edge of the species' range in Uruguay, an AMOVA was conducted to compare the two groups, and the results (data not shown) suggest that they are not different.

On average, the gene diversity within populations based on halotypes (*Hs*) was 0.6355 and the nucleotide diversity (π) was 0.0229 (Table 3). The levels of diversity did not differ greatly among the populations. Both indices indicated that the Uruguayan *C. hominivorax* populations were highly polymorphic.

Population Differentiation. The overall estimates of nucleotide divergence (δ), similarity (F), and Φ_{ST} parameters are shown in Table 3. The degree of genetic divergence of DNA sequences between two populations is expected to be correlated with the proportion of DNA fragments that they share (Nei and Li 1979). The high similarity (96.7%) and the low nucleotide divergence (δ = 0.00055) estimated for the populations agreed with this correlation and indicate that the sampled populations were very similar.

Two hierarchical AMOVA were used to investigate population differentiation: one considered the pairwise differences among populations and the other considered Nei's genetic distance. The first revealed that 92.4% of the genetic variation was attributable to the variance within populations and the second that 85.5% of this variation can be ascribed to the same hierarchical level. Although both estimates of the Φ_{ST} values among populations indicated some subpopulation differentiation, they were not statistically significant (Table 3).

Discussion

PCR-RFLP analysis of the two amplified regions of the *C. hominivorax* mitochondrial genome by using three restriction endonucleases revealed high genetic variability, with nine haplotypes in the seven Uruguayan screwworm populations sampled (Fig. 1; Table 2).

The PCR-RFLP analysis revealed marked polymorphism in the *cox*1/*cox*2 region at the intra- and interpopulational levels and was useful for defining different haplotypes. However, the A+T/12S region

revealed lower PCR-RFLP than expected. For example, only one pattern was found for 96% of the samples analyzed, indicating that the PCR-RFLP approach was not efficient in detecting variation in the A+T/12S region. This result could be attributed to the low resolution of the 2% agarose gel used to screen restriction patterns containing fragments <200 bp in size. In any case, the size variation in the A+T/12S region is an indication of length polymorphism as a molecular marker for screen intraspecific variation.

The high genetic variability was consistent with conclusions reached using independent data sets based on mtDNA RFLP for populations from North America, Central America, and Brazil, which described *C. hominivorax* as a polymorphic species (Roehrdanz 1989, Azeredo-Espin 1993, Infante-Vargas and Azeredo-Espin 1995, Taylor et al. 1996).

The nucleotide diversity (2.3%) estimated for *C. hominivorax* in this study is the highest value obtained for this species and is much higher than the same diversity previously described for Brazilian populations (0.92%) (Infante-Vargas and Azeredo-Espin 1995) and for North and Central American populations (1.3%) (Roehrdanz 1989). This high value probably reflects the inclusion of only polymorphic fragments. However, gene diversity is not affected by the choice of fragments. It can be interpreted as the probability that two randomly chosen flies in a population have different haplotypes. The average value of gene diversity estimated here ($Hs = 0.633$) indicates a high level of genetic variation for *C. hominivorax* and reinforces the results previously obtained.

Comparisons among the Uruguayan screwworm populations clearly indicated that there was no evidence of subpopulation differentiation. The presence of the common haplotype 1 at a high frequency in all populations and the wide distribution of haplotypes 3 and 4 suggested that the populations were very similar. The high value of the similarity index (96.7%) confirmed this observation. There were six local haplotypes, but the divergence between each of them and the common haplotypes was very low (just one mutational step; *d* data not shown). For this reason, the estimates of nucleotide divergence between populations was very low ($\delta = 0.00055$), indicating that the populations analyzed were very similar.

The AMOVA showed that the genetic variability was distributed mainly within populations. This finding and the Φ_{ST} estimates provide evidence that there was no genetic differentiation by natural forces, such as drift and selection, thus reinforcing the evidence that the screwworm populations of Uruguay are a unique panmictic population.

The population structure of *C. hominivorax* throughout its distribution has been a controversial topic. Allozyme studies have found no differentiation among North and Central American screwworm populations (Krafsur and Whitten 1993, Taylor and Peterson 1994) and only moderate differentiation in Brazilian populations (Infante-Malachias 1999). Infante-Malachias et al. (1999) studied *C. hominivorax* populations from southeastern Brazil and northern Argentina by using random amplification of polymorphic DNA (RAPD) and found moderate population differentiation despite the low genetic distance. The mtDNA RFLP analysis of four populations from the state of São Paulo in Brazil (Infante-Vargas and Azeredo-Espin 1995) corroborated the results obtained with RAPD and allozymes for South American populations and suggested that the populations were connected by reduced gene flow. Roehrdanz and Johnson (1988) and Roehrdanz (1989) used RFLP of mtDNA to study populations from Texas, Mexico, Jamaica, Costa Rica, and Guatemala; both studies analyzed sequence diversity among populations and concluded that there was some differentiation among the "mainland" versus Jamaican samples and less variation among samples from northern Mexico and Texas.

The distribution of mtDNA polymorphism at a geographic level among the Uruguayan screwworm populations, suggested a panmictic population in the extreme south of this species' occurrence. Based on the distribution of *C. hominivorax* mtDNA polymorphisms in the Neotropical region, we concluded that the populations of this fly cannot be differentiated at the limits of the species' distribution, in contrast to the variation found in southeastern Brazil based on RFLP of mtDNA (Infante-Vargas and Azeredo-Espin 1995). Further analyses using populations from other South America countries with emphasis in Brazilian *C. hominivorax* populations are being done to elucidate the intraspecific genetic variability in this species at a geographic level.

The lack of genetic structure among the screwworm populations in Uruguay may reflect the effect that there are no geographical barriers or important climatic differences among the regions studied. This absence of barriers would facilitate the natural dispersion of flies within Uruguay. Second, according to the Uruguayan Ministry of Agriculture (MGAP 2002), all regions of the country have a large livestock population, such that the trading of animals, possibly infected with *C. hominivorax*, could contribute to the dispersal of this species and results in more homogeneous populations.

Because our data showed that the populations of *C. hominivorax* in Uruguay form a single, panmictic and highly polymorphic population, some implications for sterile control programs need to be discussed. Roehrdanz (1989) showed that the genetic variability in mtDNA, in the form of different haplotypes, was extensive and that the sequence divergence was not great among populations from Texas, Mexico, Costa Rica, Guatemala, and Jamaica, but with greater differences between Jamaican and "mainland" populations. This extensive variability in mtDNA has not adversely affected the eradication programs because SIT has effectively eliminated the *C. hominivorax* from Mexico and from some Central American countries (Wyss and Galvin 1996). In Uruguay, we obtained similar results to those reported by Roehrdanz (1989), which suggests that this country could be a place of choice for testing the efficiency of SIT in South America. Because sterile insect release programs, either as

a holding buffer zone or an eradication campaign, require knowledge of the composition of the target species to determine optimal strategies, we are now using other molecular markers, such as microsatellite (Torres et al. 2004), to obtain a better understanding of the genetic structure of *C. hominivorax* in South America.

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