Analysis of Codon Bias in Mitochondrial Genome of *Epinephelus Fuscoguttatus*

Lirong Bai

Beibu Gulf University

Lili Lu

Beibu Gulf University

Suping Li

Beibu Gulf University

Jicui He

Beibu Gulf University

Jian Chen

Beibu Gulf University

Pei Wang

Beibu Gulf University

Yin Guo

Beibu Gulf University

Hongchen Wang

Beibu Gulf University

Dahui Yu (✉ pearlydh@163.com)

Beibu Gulf University

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1 **Analysis of Codon Bias in Mitochondrial Genome of**
2 ***Epinephelus fuscoguttatus***

3 Lirong Bai[#], Lili Lu[#], Suping Li, Jicui He, Jian Chen, Pei Wang, Yin Guo, Hongchen Wang,
4 Dahui Yu^{1*}

5 Guangxi Key Laboratory of Beibu Gulf Marine Biodiversity Conservation, Beibu Gulf

6 University, Qinzhou 535011, Guangxi, PR China

7 Lirong Bai and Lili Lu contributed equally to this work.

8 Corresponding Author: Dahui Yu

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Abstract

Background: *Epinephelus fuscoguttatus* is one of the rare marine economic fishes with high economic value. At present, the researches on grouper mainly focus on artificial propagation, physiology and biochemistry, diseases and so on. However, there are few reports on mitochondrial genome level. The research aimed to analyze composition characteristics and usage preference of codon of mitochondrial genome in *E. fuscoguttatus*, and explored main factors of affecting the formation of codon preference, thereby providing theoretical basis for studying species evolution, genetics and breeding, and improving expression efficiency of exogenous genes.

Results: GC content of mitochondrial genome of *E. fuscoguttatus* changed between 44.00% and 46.30%, with 45.40% of mean. Change range of CAI value was between 0.125 and 0.202, and the mean was 0.155. Effective number of codons (ENC) changed between 36.08 and 49.55, with 44.98 of mean. There were 32 codons that relative synonymous codon usage (RSCU) was more than 1, mainly ended with A/C. ENC-plot analysis found that all the genes were in the lower middle of the standard curve, and there was larger difference between actual and theoretical ENC, illustrating that codon bias was mainly affected by the choice. Correspondence analysis showed that the first axis contributed 58.85% of the difference, while the second, third and fourth axes contributed 14.59%, 7.66% and 5.43% of the difference respectively. Cumulative contribution rate of the first four vectors was 85.53%. Finally, nine optimal codons were selected: CUU, AUC, GUU, CCU, GCA, UAU, CGC, AGC and GGC.

Conclusions: Codon usage preference of mitochondrial genome of *E. fuscoguttatus* was weak, and

it preferred to use A/C terminated codon, and preference was mainly influenced by choice.

Key words: *Epinephelus fuscoguttatus*, Mitochondrial genome, Codon preference

1 Background

According to the genetic central dogma, each triplet codon encodes and translates an amino acid in the polypeptide chain^[1]. In eukaryotes, there are 61 triplet codons encoding 20 kinds of amino acids and three stop codons (TAA, TAG, TGA). Except methionine and tryptophan have only one codon, other 18 kinds of amino acids are encoded by 2-6 codons^[2]. Among them, codons that encode the same amino acid are called synonymous codons^[3]. The frequency of synonymous codons is different, which is called codon preference^[4]. There are many factors influencing the formation of codon usage preference, such as gene expression level, gene length, base composition, amino acid conservation, protein hydrophilicity, transcriptional selection and RNA stability^[5-7]. It is helpful to provide a theoretical basis for analyzing its gene expression, high expression of foreign genes and molecular evolution by studying codon preference of one species.

Mitochondria are important organelles in eukaryotic cells, which can provide a place for a series of life activities of cells, such as cell information transmission, energy transformation, cell differentiation and apoptosis^[8]. Mitochondrial DNA is characterized by maternal inheritance, easy amplification, high conservation and rapid evolution^[9-10]. Therefore, mitochondrial genome is widely used in genome evolution^[11], population genetics^[12], and systematic evolution^[13]. In recent years, the study on codon usage preference of mitochondrial genome has been successively reported in many animals and plants, such as *Oplegnathus punctatus* and *Oplegnathus fasciatus*^[14], *Aquarius paludum*^[15], *Eurydema dominulus*^[16], *Glycine max* and *Glycine soja*^[17], *Fusarium solani*^[18].

Epinephelus fuscoguttatus belongs to *Epinephelus*, Epinephelinae, Serranidae, Percoidae, Perciformes, Actinopterygii^[19-20], and is distributed in subtropical and tropical seas. It has the characteristics of fast growth, large specifications, delicious taste and rich nutrition, and very high economic value, and is one of the rare marine economic fishes^[21]. At present, domestic and foreign researches on *E. fuscoguttatus* mainly focus on artificial propagation, physiology and biochemistry, diseases and so on. Liao Jingqiu et al.^[22] studied karyotype structure of chromosomes of *E. fuscoguttatus*. Fan Yanjun et al.^[23] established cell lines of fin, heart and swim bladder of *E. fuscoguttatus*. Shi Gang et al.^[24] studied tolerance of *E. fuscoguttatus* to gradual and abrupt changes in salinity. Shapawi et al.^[25] studied how to optimize the growth performance of *E. fuscoguttatus* by adjusting the fat and protein contents of feed. By sequencing and analyzing brain, liver and muscle of *E. fuscoguttatus*, Lin Mingde^[26] obtained transcriptome data of brain, liver and muscle, and screened some differentially expressed genes related to growth. Most of these studies start with physiological diseases, but few studies are reported at the mitochondrial genome level. In this paper, mitochondrial gene sequence of *E. fuscoguttatus* was taken as research object for analyzing codon preference. The codon with high preference for the species was understood, and optimal codon was determined, and the factors affecting its codon preference were explored, which could provide scientific basis for studying germplasm resources, fish evolution, genetics and breeding.

2 Results

2.1 Analysis of codon composition

To more accurately analyze codon preference, nine non repetitive genes that took ATG as start codon, and sequence length of coding region was more than 300 bp in mitochondrial genome of *E. fuscoguttatus* were selected, and software Codonw was used for analyzing codon usage preference.

The results showed that GC contents on the first, second and third positions of codon changed between 46.70% and 60.00%, 32.50% and 48.30%, and 38.70% and 48.10% (Table 1), and their means were 54.30%, 39.80%, and 42.00%. GC content in the second place was lower than that in the third place, while GC content in the third place was also lower than that in the first place. ENC value changed between 36.08 and 49.55, with 44.98 of mean. Change range of CAI was between 0.125 and 0.202, and the mean was 0.155. Change range of CBI was between -0.066 and 0.043, and the mean was -0.011. FOP changed between 0.327 and 0.422, with 0.376 of mean. Gravy of protein changed between -0.917 and 1.136, and the mean was 0.685.

Table 1 Major parameters of codon in mitochondrial genome of *E. fuscoguttatus*

Gene	GC1	GC2	GC3	GC	GC3S	ENC	CAI	CBI	FOP	Gravy
ND1	0.554	0.410	0.401	0.456	0.394	40.36	0.134	-0.059	0.343	0.882
ND2	0.519	0.433	0.427	0.460	0.422	42.78	0.128	-0.060	0.341	0.875
COX1	0.548	0.396	0.426	0.457	0.414	47.08	0.202	0.020	0.412	0.901
COX2	0.600	0.325	0.395	0.440	0.387	47.17	0.191	0.034	0.414	0.468
ATP6	0.554	0.371	0.415	0.446	0.413	49.55	0.125	-0.047	0.332	1.136
ND3	0.576	0.387	0.387	0.450	0.382	36.08	0.125	-0.066	0.327	1.046
ND4	0.536	0.400	0.430	0.456	0.423	46.12	0.136	0.028	0.391	0.963
ND5	0.467	0.483	0.420	0.457	0.401	48.84	0.172	0.006	0.403	-0.917
CYTB	0.530	0.377	0.481	0.463	0.477	46.85	0.182	0.043	0.422	0.814
Mean	0.543	0.398	0.420	0.454	0.413	44.98	0.155	-0.011	0.376	0.685

Correlation analysis of codon parameters (Table 2) showed that there was extremely significant negative correlation between GC1 and GC2, and correlation coefficient was -0.900. GC1 was

negatively correlated with GC, with -0.686 of correlation coefficient. There was no significant correlation between GC2 and GC3, and GC3 was significantly correlated with GC, with 0.702 of correlation coefficient. GC3 showed extremely significant correlation with GC3S, with 0.984 of correlation coefficient, while there was no significant correlation between GC3S and CAI, CBI, Fop, ENC, Gravy. ENC was related to GC1, GC2, GC3, but it did not reach significant level, illustrating that base composition had an effect on codon bias, but it was not the main factor.

Table 2 Correlation analysis of the codon parameters

Item	GC1	GC2	GC3	GC	GC3S	ENC	CAI	CBI	FOP	Gravy
GC2	-0.900**									
GC3	-0.461	0.112								
GC	-0.686*	0.626	0.702*							
GC3S	-0.344	-0.002	0.984**	0.650						
ENC	-0.328	-0.020	0.442	-0.067	0.375					
CAI	-0.013	-0.177	0.319	0.027	0.216	0.506				
CBI	-0.081	-0.230	0.530	0.074	0.451	0.639	0.789*			
FOP	-0.712	-0.100	0.513	0.163	0.408	0.407	0.900**	0.965**		
Gravy	0.645	-0.593	0.006	-0.080	0.161	-0.367	-0.383	-0.293	-0.293	
Aromo	-0.150	0.066	0.360	0.344	0.279	0.127	0.644	0.407	0.407	-0.233

Note: * means significant correlation at 0.05 level; ** means significant correlation at 0.01 level.

RSCU is an important parameter of measuring codon usage bias^[29]. When RSCU value of codon is equal to 1, it shows that the codon has no preference. When RSCU value is more than 1, it shows that the codon is frequently used. When RSCU value is less than 1, it shows that the codon is less

used^[30]. RSCU analysis results showed that there were 32 codons that RSCU was more than 1 (Table 3). Among them, there were 15 codons ending in C, 11 codons ending in A, 6 codons ending in U, and none ending in G.

2.2 Neutral plot analysis

GC3 was taken as horizontal ordinate, and GC12 was used as vertical ordinate for correlation analysis. If there was significant correlation between GC12 and GC3, it showed that the base selection preferences of the three positions of codon were similar, and codon usage bias was affected by mutation. If there was no significant correlation between GC12 and GC3, and regression coefficient was close to 0, it illustrated that codon usage bias was influenced by selection. Neutral plot analysis of mitochondrial genes in *E. fuscoguttatus* was shown as Fig.1. In Fig.1, most of the genes were located above the diagonal, and GC12 changed between 45.35% and 48.20%, while change range of GC3 was between 38.70% and 48.10%, and the distribution range of both was small. Correlation coefficient between GC12 and GC3 was -0.648, and regression coefficient (namely slope in Fig.1) was 0.2257, which was close to 0. Bilateral test did not reach significant level ($P>0.05$), showing that correlation between GC12 and GC3 was weaker. The results showed that selection was main factor forming codon preference of mitochondrial genome in *E. fuscoguttatus*.

Fig.1 Neutrality plot analysis of mitochondrial genes of *E. fuscoguttatus*

2.4 ENC-plot analysis

ENC-plot results were shown as Fig.2. Seen from Fig.2, ENC value changed between 36.08 and 49.55, and mean was 44.98. Change range of GC3S content was between 38.20% and 47.70%, with 41.26% of mean. Standard curve in Fig.2 showed that codon usage was only affected by mutation, while the points represented by each gene were distributed in the middle and lower part of the standard curve. It illustrated that mutation had a weak effect on the formation of codon preference in the mitochondrial genome of *E. fuscoguttatus*. To more accurately reflect the difference between actual and predicted ENC values, ENC_{ratio} was calculated according to the formula (2), and frequency distribution of ENC_{ratio} was counted (Table 4). Statistical results showed that 44.44% of genes were distributed between 0.15 and 0.2, while 22.22% of genes were distributed between 0.2 and 0.25. The distribution of 11.11% genes ranged from 0.25 to 0.3, and 11.11% genes ranged from 0.3 to 0.35, and 11.11% genes ranged from 0.35 to 0.4. Therefore, it illustrated that selection was main factor in the formation of codon preference in mitochondrial genome of *E. fuscoguttatus*.

Fig.2 ENC-plot analysis of mitochondrial genes in *E. fuscoguttatus*

Table 4 Frequency distribution of ENC_{ratio} in mitochondrial genes of *E. fuscoguttatus*

Group	Number	Frequency / %
0.15~0.2	4	44.44
0.2~0.25	2	22.22
0.25~0.3	1	11.11
0.3~0.35	1	11.11
0.35~0.4	1	11.11

2.5 Correspondence analysis

Correspondence analysis results of RSCU showed that the first axis contributed 58.85% of difference, while the second, third and fourth axes contributed 14.59%, 7.66% and 5.43% of difference. Total contribution rate of the first four vectors was 85.53%. The first axis had the maximum contribution rate, while contribution rates of other axes decreased in turn, illustrating that

the first axis was main influence factor of codon preference. Correlation coefficients between the first axis and GC3, ENC were respectively 0.016 and 0.376, and there was no significant correlation. It illustrated that GC3 caused little impact on codon preference of mitochondrial genome in *E. fuscoguttatus*. To more intuitively observe codon preference, taking the first axis as horizontal ordinate and the second axis as vertical ordinate, 59-dimension spatial orthogonal axes were obtained (Fig.3). Seen from Fig.3, the distribution of each gene was scattered because of different codon preference.

Fig.3 Corresponding analysis based on RSCU

2.6 Determination of optimal codon

Taking ENC value as the standard, CDS sequences of all genes were sequenced, and then 20% of the genes were selected from each end. Highly expressed gene bank composed of ATP6 and ND5

and lowly expressed gene bank composed of ND1 and ND3 were screened out. Software Codonw1.4.2 was used to calculate RSCU values of the two banks, as well as Δ RSCU value (Table 5). Taking Δ RSCU>0.08 as the standard, 32 highly expressed codons were determined finally (codons marked by * in Table 5). Among them, three codons ended with A, 14 codons ended with U, 10 codons ended with G, and four codons ended with C.

32 highly expressed codons determined in Table 5 and 32 high-frequency codons in Table 3 were analyzed, and common codon was chosen as the optimal one. Mitochondrial genome of *E. fuscoguttatus* had nine optimal codons, and they were CUU, AUC, GUU, CCU, GCA, UAU, CGC, AGC and GGC. Among them, four codons ended with U, four codons ended with C, one codon ended with A, and there was no codon ended with G.

3 Discussion

By analyzing GC content and RSCU of mitochondrial genome in *E. fuscoguttatus*, it was found that there were 32 codons with RSCU>1, and 26 codons ended with A/C. So, *E. fuscoguttatus* preferred codons ending with A/C. The research showed that coding genes of mitochondrial protein of *Coregonus lavaretus* preferred codons ending with A/C in base composition^[29]; the codons of mitochondrial genome of Hynobiidae, *Pyricularia oryzae* Cav and *Oryza rufipogon* Griff, and NADH dehydrogenase subunit gene preferred to end with A/U^[10, 31-33]; *Acanthopagrus schlegelii* and *Pagrus major* preferred codons ending with G/C^[34]. It showed that there were differences in codon usage of mitochondrial gene among different organisms. Average GC content of mitochondrial gene of *E. fuscoguttatus* was 45.40%, which was higher than that of Hynobiidae and pathogenic bacteria of *O. rufipogon* Griff^[10, 31], and was lower than that of *C. lavaretus* was low^[29]. It illustrated that there was difference in GC content of different biological groups.

Codon preference is the result of many factors, and selection and mutation are two main causes for affecting codon preference^[4]. ENC-plot analysis results showed that mean ENC of codon of mitochondrial genome of *E. fuscoguttatus* was 41.26. Jiang et al.^[35] took 35 of ENC as distinguishing standard of bias. When ENC was more than 35, it was codon with weak preference. When ENC was less than 35, it was codon with strong preference. It illustrated that codons of mitochondrial genome in *E. fuscoguttatus* had weak preference, and CAI mean was 0.155, which also supported the opinion.

Neutral plot analysis results showed that there was no significant correlation between GC12 and GC3, and slope was smaller, illustrating that selection was main factor causing codon preference. ENC-plot analysis results also illustrated the opinion. In the chart, all the genes were located in the lower middle of the standard curve and far away from the curve, and there was larger difference between actual and theoretical ENC values. Therefore, it was thought that selection was main cause affecting codon usage preference of mitochondrial genome of *E. fuscoguttatus*. It was the same with the conclusions of more than 20 kinds of *Hynobius*, such as *Hynobius arisanensis*, *Hynobius formosanus*, *Hynobius amjiensis*^[31], and many kinds of insects, such as *Eurydema dominulus*^[16,36].

In construction of heterologous expression system, different species have different codon usage preferences^[37]. Therefore, it has important significance for optimizing and transforming genes, promoting translation efficiency, and improving expression of foreign genes to analyze codon usage patterns of species^[34]. In this paper, the combination method of codons with high expression and high frequency was used to determine the codon shared by them as the optimal one, and nine codons were finally determined as optimal codons of mitochondrial genome of *E. fuscoguttatus*: CUU, AUC, GUU, CCU, GCA, UAU, CGC, AGC and GGC. The research could provide theoretical basis

for carrying out breeding improvement of *E. fuscoguttatus* from genetic essence by optimizing and transforming genes, and improving expression efficiency of foreign genes.

4 Conclusions

Codon usage preference of mitochondrial genome of *E. fuscoguttatus* was weak, and it preferred codons ending with A or U, and the formation of codon preference was mainly affected by selection. Meanwhile, nine codons (CUU, AUC, GUU, CCU, GCA, UAU, CGC, AGC and GGC) were determined as optimal codons of mitochondrial genome of *E. fuscoguttatus*. Above analysis results could provide theoretical basis for analyzing its gene expression, high expression of foreign genes, and molecular evolution.

5 methods

5.1 Sequence source

Mitochondrial genome of *E. fuscoguttatus* was searched and downloaded from NCBI database (<https://www.ncbi.nlm.nih.gov/nuccore/MK791189.1>), and a total of 26 CDS (coding DNA sequence) were downloaded. To reduce sample error, the repetitive sequences were eliminated firstly, and then nine gene sequences with ATG as the starting codon and length greater than 300 bp were selected for analysis (Table 6).

Table 6 Length of gene sequence

Gene	Length // bp
ND1	974
ND2	1044
COX1	1550
COX2	690

ATP6	683
ND3	348
ND4	1380
ND5	1838
CYTB	1140

233 5.2 Data analysis

234 5.2.1 Analysis of codon preference parameters.

235 Software Codonw 1.4.2 was used to respectively calculate total GC content (GC), GC content of
236 synonymous codon on the third position (GC3S), relative synonymous codon usage (RSCU),
237 effective number of codon (ENC), codon adaption index (CAI), codon bias index (CBI), frequency
238 of optimal codons (FOP), grand average of hydropathy (Gravy) of DNA sequences of nine protein
239 coding genes. GC contents of codons on the first, second and third positions were showed by GC1,
240 GC2 and GC3, while the mean of GC1 and GC2 was showed by GC12.

241 5.2.2 Neutral plot analysis.

242 Neutral plot analysis is a analytic method to measure whether codon usage preference is affected
243 by mutation or selection. Neutral plot analysis took the mean GC12 calculated by GC1 and GC2 as
244 vertical ordinate, and GC3 as horizontal ordinate, to make scatter plot. In the chart, each point
245 represents an independent gene. By analyzing the relationship among the first, second and third
246 bases composition of codons, whether codon usage preference was affected by mutation or selection
247 was judged.

248 5.2.3 ENC-plot mapping

249 ENC-plot mapping took ENC as vertical ordinate and GC3S as horizontal ordinate, to make

scatter plot. Each gene was located in the scatter plot, and standard curve of ENC value was added, and standard curve equation^[27] was:

$$ENC_{exp}=2+GC3S+\frac{29}{GC3S^2+(1-GC3S)^2} \quad (1)$$

$$ENC \text{ ratio: } ENC_{ratio}=\frac{ENC_{exp}-ENC_{obs}}{ENC_{exp}} \quad (2)$$

According to scatter plot and distribution results of ENC_{ratio} , if the gene was located on or along the standard curve, the codon preference was only affected by mutation; if the gene was located far below the standard curve, the codon preference was affected by selection.

5.2.4 Correspondence analysis.

Correspondence analysis is a multivariate statistical analysis method in codon usage patterns. Via software Codonw, correspondence analysis was used for codon analysis of mitochondrial genome of *E. fuscoguttatus*, which was helpful to understand the internal law of codon distribution in mitochondrial genome of *E. fuscoguttatus*.

5.2.5 Determination of optimal codon.

$\Delta RSCU$ method^[28] was used to determine optimal codon. ENC value was sequenced, and 20% genes were selected from each end to establish gene banks of high and low expression, and then $\Delta RSCU$ of gene banks of high and low expression was calculated. If $\Delta RSCU$ was more than 0.08, and RSCU value of the codon was more than 1 in gene bank of high expression, it was determined as optimal codon.

abbreviations

RSCU: relative synonymous codon usage; ENC: effective number of codon; CAI: codon adaption index; CBI :codon bias index; FOP: frequency of optimal codons; Gravy: grand average of hydropathy; Amoro: Amoromaticity;

272 **Declaration**

273 **Ethics approval and consent to participate:**

274 All authors are willing to participate in this study and the experiment was conducted according
275 to the guidelines established by Beibu Gulf University .

276 **Consent for publication**

277 All authors are consent for publication

278 **Availability of data and materials**

279 All data are included in this manuscript; data sharing is not applicable to this article.

280 **Competing interests:**

281 The authors declare that they have no competing interests.

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290 **Authors' contributions:**

291 BLR, LLL and YDH carried out data collation, writing review, editing, funding acquisition and
292 project administration; LSP, HJC, CJ, WP, GY and WHC done data collation, writing review and
293 editing.

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295 Not Applicable

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Table 3 Relative synonymous codon usage analysis of mitochondrial genes in *E. fuscoguttatus*

Amino	Codon	Number	RSCU	Amino	Codon	Number	RSCU
Phe	UUU	77	0.92	Ser	UCU	44	1.23
	UUC	90	1.08		UCC	55	1.53
Leu	UUA	77	0.86		UCA	49	1.37
	UUG	9	0.1		UCG	5	0.14
	CUU	132	1.47		AGU	19	0.53
	CUC	128	1.42		AGC	43	1.2
	CUA	157	1.74	Pro	CCU	87	1.43
	CUG	37	0.41		CCC	83	1.36
Ile	AUU	110	1.09		CCA	61	1
	AUC	110	1.09		CCG	13	0.21
	AUA	84	0.83	Thr	ACU	61	0.93
Met	AUG	33	1		ACC	93	1.43
Val	GUU	45	1.22		ACA	96	1.47
	GUC	39	1.06		ACG	11	0.17
	GUA	49	1.33	Ala	GCU	38	0.67
	GUG	14	0.38		GCC	100	1.77
Tyr	UAU	59	1.02		GCA	84	1.49
	UAC	57	0.98		GCG	4	0.07
Cys	UGU	12	0.69	TER	UAA	12	0.38

	UGC	23	1.31		UAG	2	0.06
His	CAU	39	0.67		UGA	80	2.55
	CAC	77	1.33	Trp	UGG	16	1
Gln	CAA	72	1.62	Arg	CGU	16	0.82
	CAG	17	0.38		CGC	29	1.49
Asn	AAU	59	0.9		CGA	39	2
	AAC	72	1.1		CGG	5	0.26
Lys	AAA	63	1.73	Arg	AGA	12	0.62
	AAG	72	1.1		AGG	16	0.82
Asp	GAU	13	0.42	Gly	GGU	33	0.8
	GAC	49	1.58		GGC	58	1.4
Glu	GAA	58	1.71		GGA	52	1.25
	GAG	10	0.29		GGG	23	0.55

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429 Table 5 Relative synonymous codon usage of highly and lowly expressed gene banks of

430 mitochondrial genes in *E. fuscoguttatus*

Amino acid	Codon	Highly expressed genes		Lowly expressed genes		Δ RSCU
		Number	RSCU	Number	RSCU	
Phe	UUU**	20	1.25	13	1	0.25
	UUC	12	0.75	13	1	-0.25
Leu	UUA	13	0.66	17	1.1	-0.44

	UUG	3	0.15	3	0.19	-0.04
	CUU***	40	2.02	22	1.42	0.6
	CUC	25	1.26	19	1.23	0.03
	CUA	24	1.21	28	1.81	-0.6
	CUG**	14	0.71	4	0.26	0.45
Ile	AUU	16	1.02	15	1.07	-0.05
	AUC*	22	1.4	17	1.21	0.19
	AUA	9	0.57	10	0.71	-0.14
Met	AUG	10	1	2	1	0
Val	GUU*	8	1.39	6	1.26	0.13
	GUC	7	1.22	6	1.26	-0.04
	GUA	7	1.22	6	1.26	-0.04
	GUG	1	0.17	1	0.21	-0.04
Ser	UCU	12	1.09	6	1.33	-0.24
	UCC	10	0.91	8	1.78	-0.87
	UCA	6	0.55	10	2.22	-1.67
	UCG*	1	0.09	0	0	0.09
Pro	CCU***	50	2.02	11	1.38	0.64
	CCC	23	0.93	14	1.75	-0.82
	CCA	19	0.77	7	0.88	-0.11
	CCG*	7	0.28	0	0	0.28
Thr	ACU***	26	1.58	7	0.85	0.73

	ACC	16	0.97	14	1.7	-0.73
	ACA	19	1.15	12	1.45	-0.3
	ACG**	5	0.3	0	0	0.3
Ala	GCU***	6	1	5	0.45	0.55
	GCC	7	1.17	25	2.27	-1.1
	GCA**	10	1.67	13	1.18	0.49
	GCG*	1	0.17	1	0.09	0.08
Tyr	UAU*	26	1.33	8	1.14	0.19
	UAC	13	0.67	6	0.86	-0.19
TER	UAA***	10	1.5	1	0.27	1.23
	UAG**	2	0.3	0	0	0.3
His	CAU*	24	0.94	2	0.8	0.14
	CAC	27	1.06	3	1.2	-0.14
Gln	CAA	24	1.45	8	1.78	-0.14
	CAG**	9	0.55	1	0.22	0.33
Asn	AAU**	34	1.15	5	0.77	0.38
	AAC	25	0.85	8	1.23	-0.38
Lys	AAA	18	1.44	7	1.75	-0.31
	AAG**	7	0.56	1	0.25	0.31
Asp	GAU***	3	1.2	1	0.22	0.98
	GAC	2	0.8	8	1.78	-0.98
Glu	GAA	6	1.5	15	1.88	-0.38

	GAG**	2	0.5	1	0.13	0.37
Cys	UGU**	8	0.73	0	0	0.73
	UGC	14	1.27	1	2	-0.73
TER	UGA	8	1.2	10	2.73	-1.53
Trp	UGG	10	1	3	1	0
Arg	CGU***	7	0.61	0	0	0.61
	CGC	0	0	0	0	0
	CGA	9	0.78	9	5.4	-4.62
	CGG**	5	0.43	0	0	0.43
Ser	AGU***	15	1.36	1	0.22	1.14
	AGC***	22	2	2	0.44	1.56
Arg	AGA***	12	1.04	0	0	1.04
	AGG***	16	1.39	0	0	1.39
Gly	GGU**	4	0.94	3	0.5	0.44
	GGC**	7	1.65	8	1.33	0.32
	GGA	4	0.94	12	2	-1.06
	GGG**	2	0.47	1	0.17	0.3

431 Note: * means $\Delta\text{RSCU} > 0.08$; ** means $\Delta\text{RSCU} > 0.3$; *** means $\Delta\text{RSCU} > 0.5$.

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Figures

Figure 1

Neutrality plot analysis of mitochondrial genes of *E. fuscoguttatus*

Figure 2

ENC-plot analysis of mitochondrial genes in *E. fuscoguttatus*

Figure 3

Corresponding analysis based on RSCU