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Taxonomic status of two morphotypes of *Coryphaena hippurus* (Perciformes: Coryphaenidae)

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Two *Coryphaena hippurus* morphotypes (dourado and palombeta) are found along the Brazilian coast and are considered by Rio de Janeiro's fisherman and fishmongers as two different species. Furthermore, these morphotypes are commercialized under different values and suffer different fishing pressure. Therefore, a definition of their taxonomic status is an important economic and biological matter. In order to investigate this problem, allozyme electrophoresis method was undertaken for seventeen *loci* on 117 individuals of *C. hippurus* sampled at Cabo Frio/RJ (Brazil). The data indicate homogeneity between the morphotypes gene pools. Nevertheless, differences were found for genetic variation among dourado and palombeta, especially due to alcohol dehydrogenase *locus*. Natural selection hypothesis is discussed in explaining these findings.

Keywords: Alcohol dehydrogenase, Allozyme electrophoresis, Biochemical systematics, Fishery resources, Genetic variation.

Dois morfotipos de *Coryphaena hippurus* (dourado e palombeta) encontrados ao longo da costa brasileira são considerados espécies diferentes por pescadores e mercadores das regiões de desembarque do estado do Rio de Janeiro. Além disso, esses morfotipos são comercializados por valores diferentes e sofrem diferentes pressões de pesca. Desta forma, a definição do *status* taxonômico desses morfotipos é importante, tanto em termos econômicos quanto biológicos. A fim de investigar esse problema foi utilizado o método de eletroforese de aloenzimas com a amostragem de dezessete *loci* para 117 indivíduos dos dois morfotipos de *C. hippurus* obtidos em desembarques pesqueiros na região de Cabo Frio/RJ (Brasil). Os dados indicaram uma homogeneidade entre os conjuntos gênicos dos morfotipos. A despeito disso, diferenças entre os conjuntos gênicos de dourado e palombeta foram encontradas, devido, especialmente, ao *locus* álcool desidrogenase. A hipótese de seleção natural é discutida como possível explicação para esses resultados.

Palavras-chave: Desidrogenase do octanol, Eletroforese de aloenzimas, Recursos pesqueiros, Sistemática bioquímica, Variação gênica.

Introduction

Coryphaena hippurus Linnaeus, 1758 is an oceanic fish that has been exploited by commercial and sport fishing off the coast of Brazil (Amorim *et al*., 2011; Nóbrega *et al*., 2015). In the state of Rio de Janeiro (RJ), for example, fishing for this species reached 1,681 tons in 2012, representing 2% of the total marine fishery production in the state for that year (Fundação Instituto da Pesca do Estado do Rio de Janeiro; FIPERJ, 2013). Due to its good swimming capabilities, *C. hippurus* can be found in the epipelagic zone of tropical and subtropical waters (Gibbs Jr., Collette, 1959), and due to its very active migratory habits, the stocks for this species are expected to be very large (Díaz-Jaimes *et al*., 2010).

In parts of the Brazilian coast, fishermen at fish landings recognise two morphotypes of *C. hippurus*. In Rio de Janeiro the region, for example, the larger morphotype is referred to as dourado, whereas the smaller morphotype is known as the palombeta (designation which is also used for the species *Chloroscombrus chrysurus* occurring in coastal waters of the southwestern Atlantic). The former morphotype has a greater commercial value in the various fish markets at this location (personal observation). Therefore, defining the taxonomic status of these morphotypes is important for both commercial interests and from the point of view of fish biology, given that the management and conservation of this resource depend on the estimated size of the stocks, which in turn depends on correctly defining the morphotypes as belonging to the same species (von der Heyden *et al*., 2014).

Biochemical systematics using allozyme electrophoresis is a set of techniques that can detect biochemical differences between groups (populations, morphotypes, subspecies, etc.)

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(Moritz, Hillis, 1996; Duarte *et al*., 2016). These techniques can help resolve taxonomic issues associated with various groups of marine organisms (Knowlton, 2000), including fish (Berrebi, 2015; Nahar *et al*., 2015). This method has previously been applied to *C. hippurus* in studies of the genetic structure of stocks of the species found in the Atlantic and the Mediterranean islands (Oxenford**,** Hunte, 1986; Pla, Pujolar, 1999), as well as to define the taxonomic status of two species of the genus *Coryphaena* that are found in the Canary Islands (Pujolar**,** Pla, 2002).

In the present study, the allozyme electrophoresis method was used to evaluate the taxonomic status of two morphotypes of *C. hippurus*, which are delivered at the fish landings of Cabo Frio, RJ. The null hypothesis to be tested is that the morphotypes represent only a single species. Also, basic population genetics parameters (polymorphism, effective number of alleles, gene frequencies, and heterozygosities) are estimated for both morphotypes.

Material and Methods

Samples of one hundred and seventeen individuals of *Coryphaena hippurus* were obtained from fish landings at Cabo Frio/RJ (Brazil), between November/2013 and April/2014. Thirty two samples of dourados showed an average weight of 9345.63 g (ranging from to 4200 to 17420 g) and average total size of 128.72 cm (ranging from to 97 to 198 cm). Eighty five samples of palombetas showed an average weight of 1342.26 g (ranging from to 400 to 4090 g) and average total size of 59.18 cm (ranging from to 42 to 84 cm). Fig. 1 shows the sampling sites and respective coordinates are found in figure legend. Once landed, the specimens were dissected. Extracts of five tissues (liver, heart, eye, muscle and gonad) were placed in microtubes and kept at -20º C until the electrophoresis process.

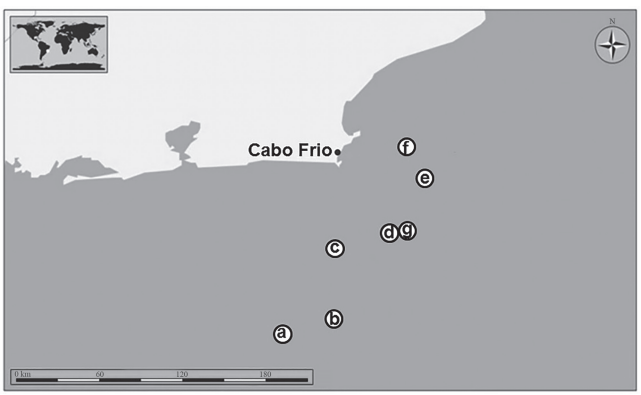

Fig. 1. Map of Cabo Frio/RJ region, with the fishing locations of the samples. **a.** 24° 04' 33.55'' S and 42° 25' 55.07'' W; **b.** 24° 01' 10.60'' S and 42° 00' 21.23'' W; **c.** 23° 30' 47.88'' S and 42° 00' 10.47''W; **d.** 23° 23' 43.60'' S and 41° 39' 41.28''W; **e.** 23° 04' 30.69'' S and 41° 19' 50.13'' W; **f.** 23° 01' 59.84'' S and 41° 26' 41.09'' W; **g.** 23° 23' 51.60'' S and 41° 39' 9.87'' W.

Horizontal gel electrophoresis was performed by standard methods using 12.5% starch gels (Harris**,** Hopinkson, 1978). The gels were stained for 30 enzyme systems using the five tissues. Eleven enzymes gave useful results for three tissues (liver, muscle and gonad). These systems were interpreted as the expression of seventeen gene *loci* (Tab. 1). The buffer systems used were discontinuous lithium hydroxide pH 8.0 (Selander *et al*., 1971) for all enzyme systems. Alleles were labelled alphabetically following decreasing electrophoretic mobility.

Tab. 1. Enzymatic systems sampled in this work, with their abbreviations, commission number (Nº EC), number of subunits (Nºsub; *1 = monomeric; 2 = dimeric; 4 = tetrameric), number of interpreted *loci*, (Nº*loci*) and tissue used for each system.

Abbreviation	Enzime	Nº EC	Nº sub.*	Nº *loci*	Tissue
α-Est	Esterase, alpha	3.1.1.1	1	3	Liver
β-Est	Esterase, beta	3.1.1.1	1	3	Gonad
G6pd	Glucose-6-phosphate dehydrogenase	1.1.1.49	2	1	Gonad
Ldh	Lactate dehydrogenase	1.1.1.27	4	1	Muscle
Mdh	Malate dehydrogenase	1.1.1.37	2	1	Muscle
Me	Malic enzyme	1.1.1.40	4	2	Gonad
Odh	Octanol dehydrogenase	1.1.1.1	2	2	Gonad
Pgi	Glucose phosphate isomerase	5.3.1.9	2	1	Gonad
Pgm	Phospoglucomutase	2.7.5.1	1	1	Muscle
Sod	Superoxide dismutase	1.15.1.1	1	1	Muscle
Xod	Xanthine oxidase	1.2.3.2	2	1	Liver

Data analyses were carried out using the software packages GENEPOP 4.2 (Rousset, 2008), BYOSIS-2 (Swofford**,** Selander, 1997), Fstat 2.9.3.2. (Goudet, 2001) and Microsoft Excel 2007 for Windows. Genetic variation was estimated at morphotype level through percentage of polymorphic *loci*, effective number of alleles (Ae; Kimura**,** Crow, 1964) and the mean number of observed and expected heterozygotes (H_{OBS} and H_{EXP} respectively) per *locus* (Nei, 1978).

Genotypic frequencies observed for each morphotype and also taking dourado and palombeta as a single group were tested for conformation to Hardy-Weinberg equilibrium using an exact test (Rousset, Raymond, 1995). The P-values obtained by the exact Markov chain method (Guo, Thompson, 1992) were corrected for multiple testing with the Bonferroni technique (Rice, 1989). Linkage disequilibrium was analyzed by performing exact tests using a Markov chain method and correcting P-values obtained with the Bonferroni technique (Rice, 1989). The null hypothesis tested was that genotypes at one *locus* are independent from genotypes at the other *locus* within each morphotype.

F-statistics analysis was used to partition genetic variation within morphotype (f) and between morphotypes (θ) components using Weir and Cockerham´s (1984)

method, which takes into account the differences in size among samples. Confidence intervals by bootstrapping over *loci* were obtained. Significance tests of F-estimates were carried out as described by Krebs (1999). The mean θ value was used to calculate the average number of migrants being exchange between the morphotypes per generation (Nm) using the equation Nm=((1/θ)-1)/4, and genetic identity were calculated according to Nei (1972).

Results

For a mean sample size of 16 individuals of the dourado morphotype and 22.8 individuals of the palombeta morphotype (overall mean of 19.4), we found an average of 2.95 alleles per *locus*, including 2.7 for the dourado morphotype and 3.2 for the palombeta morphotype. The mean polymorphism rate observed for both morphotypes was high (94.1%). Table 2 shows the gene frequencies and the expected and observed heterozygosities for each of the 17 *loci.*

Tab. 2. Allele frequencies (N = sample size for each *locus*; H_{OBS} = observed heterozygosis; H_{EXP} = expected heterozygosis).

	Morphotype	
	Dourado	Palombeta
α-Est-1		
A	0.192	0.412
B	0.808	0.500
C	0.000	0.088
N	13	17
Ho	0.385	0.294
He	0.323	0.590
α-Est-2		
A	0.060	0.280
B	0.840	0.660
C	0.100	0.060
N	25	25
Ho	0.240	0.200
He	0.287	0.492
α-Est-3		
A	0.542	0.294
B	0.167	0.647
C	0.250	0.059
D	0.042	0.000
N	12	17
Ho	0.583	0.235
He	0.641	0.506
β-Est-1		
A	0.079	0.515
B	0.842	0.439
C	0.079	0.045
N	19	33
Ho	0.158	0.303
He	0.286	0.548
β-Est-2		
A	0.156	0.406
B	0.781	0.531
C	0.063	0.063
N	16	16
Ho	0.063	0.500
He	0.373	0.567
β-Est-3		
A	0.833	0.300
B	0.167	0.300
C	0.00	0.400
N	3	5
Ho	0.333	1.000
He	0.333	0.733
G6pd		
A	0.750	0.341
B	0.250	0.341
C	0.000	0.250
D	0.000	0.068
N	4	22
Ho	0.000	0.545
He	0.429	0.717
Ldh		
A	0.966	0.867
B	0.034	0.078
C	0.000	0.011
D	0.000	0.044
N	29	45
Ho	0.069	0.267
He	0.068	0.243
Mdh		
A	0.643	0.321
B	0.238	0.393
C	0.071	0.250
D	0.048	0.036
N	21	28
Ho	0.381	0.492
He	0.535	0.691
Me-1		
A	0.833	0.889
B	0.167	0.111
N	6	9
Ho	0.000	0.222
He	0.303	0.209
Me-2		
A	0.438	0.214
B	0.563	0.571
C	0.000	0.214
N	8	7
Ho	0.375	0.571
He	0.525	0.626

Tab. 2. (continued)

	Morphotype	
	Dourado	Palombeta
Odh-1		
A	0.594	0.581
B	0.409	0.371
C	0.000	0.048
N	16	31
Ho	0.063	0.161
He	0.498	0.531
Odh-2		
A	0.864	0.947
B	0.136	0.026
C	0.000	0.026
N	11	19
Ho	0.273	0.105
He	0.247	0.104
Pgi		
A	0.441	0.569
B	0.412	0.276
C	0.088	0.121
D	0.059	0.034
N	17	29
Ho	0.647	0.759
He	0.643	0.595
Pgm		
A	0.705	0.722
B	0.136	0.130
C	0.091	0.019
D	0.045	0.037
E	0.023	0.093
N	22	27
Ho	0.409	0.333
He	0.485	0.460
Sod		
A	1.000	1.000
N	32	37
Ho	0.000	0.000
He	0.000	0.000
Xod		
A	0.194	0.286
B	0.722	0.500
C	0.083	0.214
N	18	21
Ho	0.389	0.571
He	0.446	0.638
Ho (mean)	0.257 (± 0.050)	0.382 (± 0.061)
He (mean)	0.378 (± 0.043)	0.485 (± 0.053)

However, diagnostic *loci* (Ayala**,** Powell, 1972) between morphotypes were not observed. The gene identity found among morphotypes was 0.922, whereas the estimated number of migrants per generation was 2.623. Table 3 shows the values for the inbreeding indices (F, θ and f).

Tab. 3. Inbreeding rates of Weir and Cockerham (1984) with confidence intervals for the Bootstrap averages (ns = non significant).

	F	θ	f
Values (average among *loci*)	0.308 ^{ns}	0.087 ^{ns}	0.243^{ns}
Confidence interval (95%)	[0.179; 0.428]	[0.035; 0.136]]	[0.097; 0.377]
Confidence interval (99%)	[0.139; 0.463]	[0.021; 0.152]	[0.049; 0.416]

In Table 4 are shown the probabilities of agreement with Hardy-Weinberg equilibrium for each *locus* and over all *loci* for each of the two morphotypes. Agreement with Hardy-Weinberg equilibrium was also tested taking dourado and palombeta as a single group. After the Bonferroni correction, significant differences were found only for β-*Est 2* and *Odh-1 loci* in dourado, α-*Est-2* and *Odh-1 loci* in palombeta. However, if dourado and palombeta are taken as a single group the number of loci which do not conform to Hardy-Weinberg equilibrium expectations summed up six (α-*Est-2,* α-*Est-3*, β-*Est-1*, *Mdh-3*, *Odh-1* and *Pgm*). Linkage disequilibrium was not found between the *loci*.

Tab. 4. Probability (P) of Hardy-Weinberg equilibrium (Ho: random union of gametes; *: significant before Bonferroni correction; α=0.0015625 for dourado and palombeta and α= 0.002777 for all samples).

Locus	Dourado	Palombeta	All Samples
α-*Est-1*	1.0000	0.0214	0.0222
α-*Est-2*	0.1261	0.0002*	0.0000*
α-*Est-3*	0.0376	0.0144	0.0007*
β-*Est-1*	0.0206	0.0077	0.0000*
β-*Est-2*	0.0006*	0.8136	0.0070
β-*Est-3*	-	1.0000	1.0000
G6pd	0.1429	0.3627	0.0720
Ldh	1.0000	1.0000	1.0000
Mdh	0.1826	0.0182	0.0008*
Me-1	0.0909	1.0000	0.2031
Me-2	0.5301	0.5152	0.1662
Odh-1	0.0005*	0.0000*	0.0000*
Odh-2	1.0000	1.0000	1.0000
Pgi	1.0000	0.0581	0.2652
Pgm	0.0124	0.0019	0.0000*
Sod	-	-	-
Xod	0.4124	0.4197	0.4233
All *loci*	0.0000*	0.0000*	0.0000*

Discussion

The expected and observed heterozygosity values and the degree of polymorphism found for the dourado and palombeta morphotypes in this study can be considered high compared to values usually sampled in fish using allozymes as molecular markers (Ward *et al*., 1994). Moreover, Pla AND Pujolar (1999) analysed the genetic variation for 735 young specimens of *C. hippurus* from

six locations in the Mediterranean Sea and the Atlantic Ocean using allozyme electrophoresis and observed low heterozygosity (mean of 0.0425 for the six populations sampled) and a low polymorphism rate (23.33% of the *loci* of the six populations). The difference between the levels of genetic variation found in the present study and those found by Pla and Pujolar (1999) can be explained by the *loci* that were sampled. In the study by Pla AND Pujolar (1999), of the 30 *loci* sampled, 22 were monomorphic, whereas in the present study, only one of the 17 *loci* sampled had only one allele, and the two studies only sampled three enzyme systems in common (*Ldh*, *Mdh*, and *Sod*). Some enzymes are known to exhibit more polymorphisms than others (Ward *et al*., 1992), which is the case in enzyme groups such as esterases and phosphoglucomutases. This situation applies to enzymes such as *α-Est*, *β-Est,* and *Pgi*, which were used in this study and were not part of the Pla and Pujolar (1999) study. Therefore, the difference in the set of *loci* sampled between the two studies most likely explains the higher levels of genetic variation found in this study for the species *C. hippurus*.

The results related to the taxonomic classification of the morphotypes suggest homogeneity among the groups analysed. By analysing the genetic variation of different samples of the genus *Coryphaena* captured in the Canary Islands using allozyme electrophoresis, Pujolar and Pla (2002) recognised 11 diagnostic *loci* and found two species of *Coryphaena* at that location (*C. hippurus* and *Coryphaena equiselis* Linnaeus, 1758). Other studies using the allozyme electrophoresis method found diagnostic *loci* between morphotypes of *Polynemus paradiseus* Linnaeus, 1758 (Nahar *et al*., 2015) and *Salmo trutta* Linnaeus, 1758 (Berrebi, 2015), for example, but this was not the case in the present study.

The genetic identity value (I) found between the morphotypes was equivalent to values usually found for conspecific populations (Thorpe, 1983). Other studies have estimated the gene identity between fish groups using the allozyme electrophoresis method and have associated values of I greater than 0.85 within populations of the same species (Zawadzki *et al*., 2008; Erdoğan *et al*., 2009).

The number of migrants per generation between morphotypes also indicates homogeneity between the groups analysed. Some authors (Wright, 1931; Hartl**,** Clark, 2007) have noted that only one migrant per generation may be sufficient to preserve the homogeneity among gene pools and prevent geographical differentiation, and fish allozyme data have corroborated this conclusion (Lacson, 1992; Geertjes *et al*., 2004).

The mean values for the inbreeding rates may be considered high, although not significant, indicating a lack of preferential crosses within the morphotypes (Waples, 1998). This is a common pattern observed in conspecific fish populations analysed using allozyme electrophoresis (Waples, 1987).

The finding that deviations related to Hardy-Weinberg expectations when all sampled individuals were analyzed as a single group (Tab. 4) indicates a possible temporal Wahlund effect (Johnson, Black, 1984). Individuals of the palombeta morphotype are smaller in size (lower mean weight and smaller length), have less developed gonads (that weigh less than the dourado morphotype; data not shown), and have less advanced maturation stages (most of the individuals in non-reproductive stages, based on Beardsley Jr., 1967; data not shown) than individuals of the dourado morphotype (most of the individuals in sexual maturity stage based on Beardsley Jr., 1967; data not shown). Although there was no information about the age of the fish examined, sampled palombeta corresponds in size to averaged values for juveniles of the species *C. hippurus* (Potoschi *et al*., 1999), which is below the minimum size permitted for capturing dourado (IBAMA, 2003). Therefore, taken together genetic and biological data give indications that dourado and palombeta are different life stages of *C. hippurus*. Furthermore, differences were observed between the expected heterozygosity, observed heterozygosity, and effective number of alleles for the morphotypes. Within the 10% of the sampled *loci* which showed significant deviations from the expected Hardy-Weinberg equilibrium (twice than expected), is included the *Odh-1 locus* for both morphotypes. Several studies (Hochachka, 1980; Vornanen *et al*., 2009; Torres *et al*., 2012) have noted the possibility of natural selection acting on this *locus* in fish.

Hypotheses that involve natural selection acting on the gene frequencies of one or more *loci* depend on the confirmation of various conditions (Clarke, 1975; Silva, 2009). First, it must be shown that the change in gene frequencies of the *locus* under selection cannot be explained by simple chance. In addition, the *locus* under selection should be correlated with some environmental factor. Furthermore, disequilibriums in the linkage between the *loci* in question should also be evident. Moreover, this pattern needs to be recognised in other evolutionary units (in this case, in other species with morphotype differences). The different alleles in question should produce different phenotypes, and these differences should make sense environmentally and have an impact on adaptation.

The differences in allele frequencies and in the number of *Odh* alleles between the morphotypes appear to meet the first two conditions. *Odh* (also referred to in the literature as *Adh*, No. E.C. 1.1.1.1, alcohol NAD+ oxidoreductase) is an enzyme that participates in one of the pyruvate pathways, catalysing its reduction to ethanol (rather than lactate) in the absence of oxygen. This process helps avoid lactate accumulation in the body during anaerobic metabolism, which prevents the accumulation of this compound and the resulting muscle fatigue (Shoubridge**,** Hochachka, 1980). This pathway for transforming pyruvate into ethanol is found in the swimming muscles of some fish that live in O_2 poor environments such as *Carassius carassius* Linnaeus, 1758 and *Carassius auratus* Linnaeus, 1758 (Vornanen *et al*., 2009).

The significant differences in the *Odh* enzyme detected between the dourado and palombeta morphotypes may be associated with the metabolism of their explosive swimming, which could be part of escape behaviour. Summing up the evidences of smaller size, less developed gonads and maturation of palombeta in relation to dourado morphotype and the evidence of genetic homogeneity among the morphotypes, give support to the hypothesis that palombeta may be a young stage of the dourado morphotype, which is possibly the adult stage of *C. hippurus*. Furthermore, natural selection could be acting on the variation observed in *Odh* in the species, selecting over time those alleles that have a greater efficiency in converting pyruvate to ethanol rather than lactate in the swimming muscles, resulting in a decrease in the variation of this *locus*.

An alternative hypothesis to explain the significant differences in variation found among morphotypes is the differential expression of allozymes during the life stages of the individuals. However, this is unlikely, since no enzymes were found to be exclusive of any morphotype among the sampled *loci* and literature reports correlations among *loci*-tissues-age expression rather than differential allele's expression. Therefore, it is more conservative to interpret the differences found in genetic variation among morphotypes as a result of different gene pools rather than differential expression of any allozymes.

Returning to the conditions required to the hypotheses of natural selection, linkage disequilibrium was not observed among the *loci* analysed. The fourth condition (pattern be recognised in other evolutionary units), however, appears to have been met based on the study data. Most vertebrates, including fish, depend almost exclusively on aerobic metabolism. A switch to anaerobic pathways only occurs during periods of increased physical activity or low environmental oxygen levels (Torres *et al*., 2012). Tuna, for example, appear to have mechanisms that are very well adapted to anaerobic situations, which are used during a burst of swimming activity (Hochachka, 1980), and this may also be the case for *C. hippurus*.

The last three conditions (different alleles produce different phenotypes, they make sense in the environment and have an impact on adaptation) could not be tested in this study, which weakens the hypothesis of natural selection. Therefore, the absence of important evidence such as linkage disequilibrium and the inability to test some important conditions for the hypothesis of natural selection lead us not to reject the null hypothesis as the explanation for the differences in heterozygosity and the effective number of alleles found between the morphotypes. In other words, the differences observed can be explained by chance alone.

In short, the lack of a diagnostic *locus*, high genetic identity values, number of migrants per generation, and non-significant values for the inbreeding rates indicate the absence of preferred crossings within the morphotypes. Therefore, the null hypothesis that the morphotypes belong to the same species cannot be excluded. The amount of observed variation can be considered high in relation to that generally estimated for fish and for *C. hippurus*. This finding can be explained by the cosmopolitan and migrant behaviour of this species and the *loci* sampled. The differences in heterozygosity, both expected and observed, and in the effective number of alleles between the morphotypes may be related to the action of directional natural selection at the *Odh locus*. However, due to lack of directly related evidence needed to test this hypothesis and the limited parsimony, it was not possible to accept this hypothesis; instead, we failed to reject the null hypothesis that the differences are due to chance actions.

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