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Original Research

# Comparison of Sequence Variants in the *PDK4* and *COX4I2* Genes Between Racing and Cutting Lines of Quarter Horses and Associations With the Speed Index

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ABSTRACT

Different selection objectives within the Quarter Horse breed led to the formation of groups with distinct skills, including the racing and cutting lines. With a smaller population size in Brazil, but of great economic representativeness, the racing line is characterized by animals that can reach high speeds over short distances and within a short period of time. The cutting line is destined for functional tests, exploring skills such as agility and obedience. Although the athletic performance of horses is likely to be influenced by a large number of genes, few genetic variants have so far been related to this trait and this was done exclusively in Thoroughbreds, including the g.38973231G>A single-nucleotide polymorphism in the *PDK4* gene and the g.22684390C>T single-nucleotide polymorphism in the *COX4I2* gene. The results of the present study demonstrate the presence of polymorphic *PDK4* and *COX4I2* genes in Quarter Horses. The analysis of 296 racing animals and 68 cutting animals revealed significant differences in allele and genotype frequencies between the two lines. The same was not observed when these frequencies were compared between extreme racing performance phenotypes. There were also no significant associations between alleles of the two polymorphisms and the speed index. These results suggest that the alleles of the *PDK4* and *COX4I2* genes, which are related to better racecourse performance in Thoroughbreds, are probably associated with beneficial adaptations in aerobic metabolism and therefore play secondary roles in sprint racing performance in Quarter Horses, which is mainly anaerobic.

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## 1. Introduction

During the formation of the Quarter Horse breed, animals were selected with different objectives which resulted in groups with different skills or abilities, such as the racing and cutting lines [1]. Quarter Horse racehorses show a better performance in short-distance races than any other line or breed and are the fastest horses and one of the fastest animals in the world. The Quarter Horse can reach a speed of up to 88 km/hr and can sprint the quarter mile (approximately 402 m) from a standing position in less than 21 seconds [2]. The cutting line is destined for functional tests, exploring skills such as agility and obedience, which are important for cattle management in the field. A cutting horse should be able to perceive and anticipate the movements of cattle to be a good sorting horse [3].

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The athletic potential of mammals is influenced by the complex interrelationship between a set of genes and environmental factors [4,5]. The contribution of genetics to athletic potential is well documented in humans, in which more than 220 genes have been described [6]. Although the athletic performance of horses is probably also influenced by a large number of genes, few genetic variants have so far been related to this trait and this was done exclusively in Thoroughbreds. These variants include single-nucleotide polymorphisms (SNPs) in the pyruvate dehydrogenase kinase, isozyme 4 (*PDK4*: mapped at equine chromosome 22—ECA22) gene [7,8] and in the cytochrome c oxidase (COX), subunit 4, isoform 2 (*COX4I2*: mapped at equine chromosome 4—ECA4) gene [9].

The regulation of glucose utilization is tightly controlled by the uptake of glucose by their transporters, the rate of glycolytic flux, and the conversion of pyruvate to acetyl-CoA in the mitochondria through the catalytic function of the pyruvate dehydrogenase complex (PDC). The critical rate-limiting step of glucose oxidation is the regulation of PDC assembly, which is controlled by pyruvate dehydrogenase kinase. Pyruvate dehydrogenase kinase blocks the formation of the PDC, which results in the beta-oxidation of fatty acids to acetyl-CoA as the substrate for oxidative phosphorylation. The oxidation of fatty acids is highly efficient in the generation of adenosine triphosphate (ATP) and is controlled by the expression of *PDK4* in skeletal muscle during and after exercise [10].

Cytochrome c oxidase is a multisubunit enzyme (complex IV) which catalyzes the transfer of electrons from reduced cytochrome c to oxygen during mitochondrial respiration. Cytochrome c oxidase complex IV, which is encoded by nuclear DNA, is responsible for the regulation and assembly of mitochondrially encoded subunits on the mitochondrial membrane and has been associated with mitochondrial volume. Cytochrome c oxidase complex IV consists of two isoforms (COX4-1 and COX4-2) that are encoded by the *COX4I1* and *COX4I2* genes. The two genes are differentially regulated in normoxic (normal oxygen) and hypoxic (lack of oxygen) environments. The *COX4I1* gene is preferentially transcribed in normal oxygen environments. In limited oxygen environments, the master regulator of the response to hypoxia, hypoxia inducible factor 1, activates the transcription of *COX4I2* and of the mitochondrial *LON* gene, which inhibits the expression of *COX4I1* [11].

In view of the role of the genes described in the physiology of skeletal muscle and considering that the effects of DNA polymorphisms on phenotypes are intrinsic parameters of each line or breed in a given environment, the objectives of the present study were (1) to compare the frequencies of the *PDK4* g.38973231G>A and *COX4I2* g.22684390C>T SNPs (EquCab 2.0, [12]) between the racing and cutting lines of Quarter Horses and between animals with extreme racing performance phenotypes and (2) to perform an association analysis of these polymorphisms with the speed index (SI), a quantitative trait indicative of the racing performance of Quarter Horses. The results of this study may contribute to marker-assisted selection of Quarter Horses for better racecourse performance.

## 2. Materials and Methods

### 2.1. Animals and Performance Data

All animal procedures were performed according to Brazilian guidelines of animal well-being (Protocol No. 204/2012-CEUA issued by the Ethics Committee on Animal Use of the School of Veterinary Medicine and Animal Science, UNESP, Botucatu, São Paulo, Brazil).

For this study, 364 Quarter Horses of both sexes, born between 1982 and 2011 and registered at the Brazilian Association of Quarter Horse Breeders (Associação Brasileira de Criadores de Cavalo Quarto de Milha), were used. Of these, 296 animals were of the racing line and 68 of the cutting line. The racing animals, including 67 males and 229 females born to 95 stallions and 240 mares, were housed at the Sorocaba Jockey Club and on 14 other properties in the state of São Paulo, Brazil. The cutting horses, including 26 males and 42 females born to 44 stallions and 64 mares, were housed on three properties in the state of São Paulo. Blood was collected on the horse farms in the state of São Paulo and at the Sorocaba Jockey Club. The presence of full-sibs in the two lines was avoided.

Performance data were obtained from the Department of Statistics of the Sorocaba Jockey Club and from the Equibase online database [13]. The performance record is given by the maximum SI obtained along the competition history of each animal. According to Evans [14], the SI was created for Quarter Horse races to permit the comparison of performances between animals under different conditions (distances, racetrack, climate, and country). Every year each racetrack creates its own SI table, which is derived from the average of three wins (top three times) for each of the last three consecutive years in each distance, and the average value of those nine times is equivalent to an SI of 100, creating a scale. Therefore, SI points are integers and vary with time (longer times lead to lower indices and vice versa), the level of hundredths of a second, and are adjusted by the distance traveled in the race. The SI of several years was available for most of the animals used, and the mean SI was thus calculated. However, only the maximum SI was available for other animals. Because the mean SI showed a high correlation with maximum SI ($r = 0.8762$), the latter was used to prevent the loss of performance data. The mean and standard deviation of maximum SI considering 267 animals of the racing line with the data were $95.78 \pm 7.80$.

### 2.2. Blood Collection, DNA Extraction, and Genotyping of Animals

Whole blood (5 mL) was collected from each animal by puncture of the left jugular vein in the neck region into vacuum tubes containing 7.5 mg EDTA. DNA was extracted using the Illustra Blood GenomicPrep Mini Spin Kit (GE Healthcare, USA) according to the manufacturer instructions.

For determination of the allele and genotype frequencies and for association analysis of the *PDK4* and *COX4I2* gene polymorphisms with performance, 364 Quarter Horses (racing line, n = 296; cutting line, n = 68)

**Table 1**
Description of the observed and expected band patterns of the different genotypes of the SNPs studied.

| SNP (Gene) | Genotype | Observed or Expected Band Pattern |
|---|---|---|
| g.38973231G>A (*PDK4*) | AA | 356 and 235 bp |
| | AG | 356, 235, and 177 bp |
| | GG | 356 and 177 bp |
| g.22684390C>T (*COX4I2*) | CC | 556 bp |
| | CT | 556, 291, and 265 bp |
| | TT | 291 and 265 bp |

Abbreviation: SNP, single-nucleotide polymorphism.

were genotyped. The g.38973231G>A SNP genotypes of the equine *PDK4* gene, located in intron 2, were identified for the first time by the ARMS-PCR method, using the primers: forward inner GCA GCA GTA AAG ACT ATG GAT TGA CTG; reverse inner CCA TTA AAC AAT GAC AAT CTG AAA CAA AT; forward outer GAT GCA ACT TTA ACC CTC AAC TTT CTA A; and reverse outer CAG ATT TTC AGA GAA TAG AGC CAG GAT A. In this method, allele-specific amplification of 235- and 177-bp fragments occurs for alleles A and G, respectively. The C and T alleles of the g.22684390C>T SNP of the equine *COX4I2* gene, located in intron 2, were identified for the first time by PCR-RFLP, in which a 556-bp fragment was amplified using the forward primer CCC CCA AAT ACT GAA TGC AC and reverse primer GCC AGG AGC TAG TGA CAA GG and digested with the *Xce*I restriction enzyme (Thermo Scientific, USA).

### 2.3. Allele and Genotype Frequencies of the SNPs Studied

The allele and genotype frequencies of each genotyped polymorphism were calculated from the total of 364 genotypes identified on the electrophoresis gels as described by Weir [15]. The allele and genotype frequencies of the *COX4I2* and *PDK4* genes were compared using additive model between the cutting and racing lines and using additive, dominant, and recessive models between extreme racing performance phenotypes given by the lowest and highest SI (lower and upper SI). For comparison between lower and upper SI, with means and standard deviations of 79.06 ± 3.18 and 108.20 ± 2.04, respectively (different with $P < .0001$), the phenotypes were adjusted for systematic effects of the environment (fixed effects), sex, interaction between racetrack (1–14), and distance (228, 275, 301, 320, 365, 402, and 502 m) and interaction between year of race (1988–2013) and age of animal at racing (2, 3, and 4 years). The significance of the effect of sex and of the interactions was tested using the PROC GLM procedure of the SAS v.9.1 program [16]. Hardy–Weinberg equilibrium for the racing line and differences in alleles and genotype frequencies were analyzed by the $\chi^2$ test using the R genetics package [17].

### 2.4. Association Between the SNPs Studied and Performance of the Racing Line

Association analysis was performed with 267 horses of the racing line with mixed models using the PROC MIXED procedure of the SAS v.9.1 program [16]. The model for each SNP included the random effect of sire, the fixed effects of sex, interaction between age class and year of race, and interaction between racetrack and distance, in addition to a random error. The copy number of a particular allele of the polymorphism genotype was included in the model as a covariate. Because SNPs are biallelic, that is, they only have two possible alleles, regression was performed on the most frequent allele.

## 3. Results

The electrophoretic patterns of the genotypes of the *PDK4* and *COX4I2* polymorphisms are shown in Table 1. Table 2 lists the allele and genotype frequencies of the *PDK4* g.38973231G>A and *COX4I2* g.22684390C>T SNPs in the racing and cutting lines. For the *PDK4* g.38973231G>A polymorphism, significant differences in genotype ($P = .0005$) and allele ($P = .0001$) frequencies were observed

**Table 2**
Comparison of genotype and allele frequencies of the *PDK4* and *COX4I2* and polymorphisms between the racing and cutting lines of Quarter Horses and Hardy–Weinberg equilibrium for the racing line.

| | Racing Line (n = 296) | | Cutting Line (n = 68) | | $\chi^2$ | *P* Value | HWE (*P* Value) |
|---|---|---|---|---|---|---|---|
| | n | Frequency | n | Frequency | | | |
| *PDK4* (n = 364) | | | | | | | |
| Genotypes | | | | | | | |
| GG | 141 | 0.48 | 50 | 0.74 | 15.239 | .00049 | .0358 |
| GA | 137 | 0.46 | 17 | 0.25 | | | |
| AA | 18 | 0.06 | 1 | 0.01 | | | |
| Alleles | | | | | | | |
| G | 419 | 0.71 | 117 | 0.86 | 14.62 | .00013 | |
| A | 173 | 0.29 | 19 | 0.14 | | | |
| *COX4I2* (n = 364) | | | | | | | |
| Genotypes | | | | | | | |
| CC | 102 | 0.34 | 20 | 0.29 | 8.428 | .01479 | .0013 |
| CT | 166 | 0.56 | 33 | 0.49 | | | |
| TT | 28 | 0.09 | 15 | 0.22 | | | |
| Alleles | | | | | | | |
| C | 370 | 0.62 | 73 | 0.54 | 3.253 | .07127 | |
| T | 222 | 0.38 | 63 | 0.46 | | | |

Abbreviations: $\chi^2$, chi-squared; HWE, Hardy–Weinberg equilibrium.

**Table 3**
Comparison of genotype and allele frequencies of the *PDK4* and *COX4I2* gene polymorphisms between Quarter Horses with extreme racing performance phenotypes.

| | Upper SI (n = 20) | | Lower SI (n = 20) | | $\chi^2$ | *P* Value |
|---|---|---|---|---|---|---|
| | n | Frequency | n | Frequency | | |
| *PDK4* | | | | | | |
| Genotypes | | | | | | |
| GG | 10 | 0.5 | 11 | 0.55 | 1.114 | .5728 |
| GA | 7 | 0.35 | 8 | 0.4 | | |
| AA | 3 | 0.15 | 1 | 0.05 | | |
| Alleles | | | | | | |
| G | 27 | 0.68 | 30 | 0.75 | 0.244 | .6213 |
| A | 13 | 0.32 | 10 | 0.25 | | |
| Dominant | | | | | | |
| GG | 10 | 0.50 | 9 | 0.45 | 0 | 1 |
| A_ | 10 | 0.50 | 11 | 0.55 | | |
| Recessive | | | | | | |
| G_ | 3 | 0.15 | 1 | 0.05 | 0.278 | .5982 |
| AA | 17 | 0.85 | 19 | 0.95 | | |
| *COX4I2* | | | | | | |
| Genotypes | | | | | | |
| CC | 6 | 0.3 | 11 | 0.55 | 2.56 | .2871 |
| CT | 11 | 0.55 | 7 | 0.35 | | |
| TT | 3 | 0.15 | 2 | 0.1 | | |
| Alleles | | | | | | |
| C | 23 | 0.57 | 29 | 0.72 | 1.374 | .2412 |
| T | 17 | 0.43 | 11 | 0.28 | | |
| Dominant | | | | | | |
| CC | 6 | 0.30 | 11 | 0.55 | 1.637 | .2008 |
| T_ | 14 | 0.70 | 9 | 0.45 | | |
| Recessive | | | | | | |
| C_ | 17 | 0.85 | 18 | 0.90 | 0 | 1 |
| TT | 3 | 0.15 | 2 | 0.10 | | |

Abbreviations: $\chi^2$, chi-squared; A_, AA and AG; C_, CC and CT; G_, GG and GA; SI, speed index; T_, TT and TC.

between the racing and cutting lines, with the A allele, which is favorable to racecourse performance in Thoroughbreds [8], being more frequent in the racing line. There was a significant difference in the genotype frequencies of the *COX4I2* g.22684390C>T SNP between lines. Although not significant ($P = .071$), the T allele, which is favorable to racecourse performance in Thoroughbreds [9], was found at a lower frequency in the racing line compared with the cutting line (0.38 vs. 0.46). Testing the results of Table 2 for Hardy–Weinberg equilibrium showed that the *PDK4* ($P = .0358$) and *COX4I2* ($P = .0013$) polymorphisms studied deviated from Hardy–Weinberg proportions in the Quarter Horse racehorse population of the state of São Paulo. Comparisons of the allele and genotype frequencies of the *PDK4* and *COX4I2* gene SNPs between animals with extreme racing performance phenotypes (Table 3) revealed no significance differences ($P > .05$).

The significance of the fixed effects of sex and of the interactions between racetrack and distance and between year of race and age of animal at racing used in the association model between the *COX4I2* and *PDK4* SNPs and performance was 0.0009, 0.0001, and 0.362, respectively. Effect o allele substitution analysis revealed no significant associations between the *PDK4* or *COX4I2* polymorphism and maximum SI (Table 4). The A to G substitution in the *PDK4* gene and the T to C substitution in the *COX4I2* gene were considered.

**Table 4**
Allele substitution effect of the *PDK4* g.38973231G>A and *COX4I2* g.22684390C>T SNPs on the racing performance of Quarter Horses.

| Gene | n | Substitution | Effect | *P* Value |
|---|---|---|---|---|
| *PDK4* | 267 | G>A | −1.5991 | .1104 |
| *COX4I2* | 267 | T>C | −0.8788 | .3666 |

Abbreviation: SNP, single-nucleotide polymorphism.

## 4. Discussion

The genotyping protocols using ARMS-PCR for the *PDK4* g.38973231G>A polymorphism and PCR-RFLP for the *COX4I2* g.22684390C>T SNP were found to be efficient, robust, inexpensive, and adequate for laboratories with a basic infrastructure of equipment and reagents. The efficiency of genotyping by ARMS-PCR and PCR-RFLP was confirmed by direct sequencing of PCR products of 30 animals of the total sample with different genotypes for both polymorphisms.

The deviation from Hardy–Weinberg equilibrium observed for the *PDK4* g.38973231G>A and the *COX4I2* g.22684390C>T SNPs in the racing line of Quarter Horses suggests the absence of some conditions necessary to fulfill the requirements of this theorem. Possibly, the deviation is due the introduction of animals of other breeds as sires and/or nonrandom mating and not due to selection for performance in order that the association results between genetic variants and the SI were not significant.

A variant in the *PDK4* gene was the first example of a significant association between an SNP and superior

racecourse performance in horses [18]. In the study of Hill et al [8], higher frequencies of the A allele of the *PDK4* g.38973231G>A SNP were observed in Thoroughbreds with best racing performance (0.44) compared with the group with poor performance (0.29). In the present study, higher frequencies of the A allele were observed in the racing line (0.29) compared with the cutting line (0.14). However, there were no significant differences between extreme racing performance phenotypes, and the polymorphism variants were not significantly associated with SI. Although not associated with better racing performance in Quarter Horses, the *P* value of .1104 (Table 4) and the differences in frequency between lines ($P = .00013$: Table 2) and extreme phenotypes (upper SI: 0.32; lower SI: 0.25; Table 3) may suggest that the A allele is being selected. On the other hand, and considering that the frequency of 0.29 may be low for an allele in process of selection, these results may be explained by the use of Thoroughbred horses with good performance in short-distance races as sires of the Quarter Horse racing line. This fact may increase the frequency of allele A in the breed that would not necessarily be related to better performance.

The present results showed a lower frequency of the T allele of the *COX4I2*g.22684390C>T SNP in the racing (0.38) and cutting (0.46) lines of Quarter Horses when compared with Thoroughbred horses. In the study of Gu et al [9] involving the English breed, the frequency of the T allele was significantly higher in the group with best racecourse performance (0.68) compared with the group with poor performance (0.55). Here, the T allele was more frequent (close to significance) in the cutting line than in the racing line. These results suggest that this variant may be related to improvement of aerobic metabolism in sport tests performed by cutting Quarter Horses. This allele has also been associated with best racecourse performances in Thoroughbreds [9], which run significantly longer distances even in short-distance races than those run in Quarter Horse races.

The results of the studies of allele and genotype frequencies and association presented here suggest that the SNPs g.38973231G>A in the *PDK4* gene and g.22684390C>T in the *COX4I2* gene have no relation to athletic performance of racehorses of Quarter Horse breed. It should be noted that, with the attempt to control genetic background, these same analyzes were performed with the exclusion of half-sibs and did not show important differences in significance. In fact, according to Pilegaard and Neufer [10] and Fukuda et al [11], the protein products of *PDK4* and *COX4I2* genes are closely related to aerobic energy metabolism in mammals. Some aspects of the physiological processes related to optimal athletic performance have been shown to be similar in humans and horses [19, 20]. Horses can perform different types of physical exercise that use from predominantly aerobic to predominantly anaerobic metabolism. The short-distance races of Quarter Horses are characterized by the predominant use of anaerobic muscle metabolism. In contrast, in some classes of arena exercise such as reining and sorting, modalities performed by cutting Quarter Horses, short bouts of anaerobic exercise are intercalated with longer periods of aerobic activity [21], as also observed in long-distance Thoroughbred racing.

## 5. Conclusions

Although the present results showed differences in the genotype and allele frequencies of the *PDK4* g.38973231G>A and *COX4I2* g.22684390C>T polymorphisms between the racing and cutting lines of Quarter Horses, they could not be associated with better racecourse performance. These findings suggest that these genes are related to improvement of aerobic metabolism, being less important for sprints of Quarter Horses, or that the favorable alleles were introduced by the use of Thoroughbreds sires, without the increase in their frequencies in fact contributing to the improvement of this trait. Therefore, these polymorphisms cannot be used for marker-assisted selection of animals with superior racing performance in the Quarter Horse breed, which have muscle energy metabolism predominantly anaerobic.

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