Genetic and environmental risk factors for vitiligo and melanoma in Pura Raza Español horses

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Keywords: dermatology, heritability; inbreeding; prevalence; Spanish Purebred horse

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Ethical animal research

The study was approved by the Ethical Committee of the University of Seville (Spain). A representative of the National Association of Pura Raza Español Horse Breeders (ANCCE) gave explicit informed consent for use of their data in this study.

Authors' declaration of interests

No competing interests have been declared for any authors.

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Authorship

M. Valera and M.J. Sánchez performed the study design. M.J. Sánchez, M. Valera were involved in the execution, as well as preparation of the manuscript. P.J. Azor was involved in the data collection. M. Solé participated in the analysis of data. J. Sölkner contributed to presentation and discussion of data also corrected the language. All the authors read and approved the final version of the manuscript.

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Summary

Background: Vitiligo and melanoma are relatively common disorders in grey Pura Raza Español horses (PRE) and horse breeds with grey-coloured coats.

Objectives: To determine the breed prevalence, environmental risks factors and estimate the genetic parameters for vitiligo and melanoma in PRE.

Study design: Retrospective cohort study.

Methods: We analysed data from a large worldwide population of PRE. The database included the vitiligo and melanoma scores, on either a four- or six-point linear scale, of 11,436 horses. Genetic parameters were estimated using a Bayesian genetic animal model including the four associated environmental risk factors as systematic effects. Inbreeding was used as a covariate, and animal and residual effects were included as random effects.

Results: Of the horses included in the study, 2.8% and 20.5% showed some traces of vitiligo around the eyes and mouth, respectively, while 1.6% showed varying degrees of melanoma. Age, coat colour and inbreeding were significantly associated with the three outcomes studied. The estimated heritability for the whole population was 0.09 (standard deviation, s.d. +0.019), 0.44 (s.d. +0.031) and 0.13 (s.d. +0.037), for eye vitiligo score, nostril vitiligo score and melanoma scores respectively. The genetic correlations ranged from 0.42 (s.d. +0.084) between eye and nostril vitiligo score to 0.15 (s.d. +0.096) between nostril vitiligo and melanoma.

Main limitations: Vitiligo scores for the perianal regions were not collected. The veterinarian responsible for each assessment was not recorded.

Conclusions: Vitiligo and melanoma are prevalent in this population and those environmental risk factors and genetics both have an effect on the clinical expression of the diseases. These findings may help to reduce prevalence through breeding programmes.

Introduction

Vitiligo is an acquired, non-contagious disorder, characterized by progressive patchy loss of skin pigmentation. Vitiligo tends to cover hair and mucous membranes (due to a loss of melanocytes in the affected areas) [1]. Despite the fact that the phenotype of vitiligo has been readily recognized for thousands of years, the underlying pathobiology has remained largely unknown [2]. Similarly, melanomas are usually pigmented neoplasm which often present multicentric malignancy in the later stages [3]. They are most commonly found in grey horses and reach a prevalence of around 80% in horses of 15 years of age or older [4]. Studies in humans indicate that there is a genetic link between melanoma and vitiligo [5,6]. Moreover, in horses, the dominant phenotype of greying with age is associated with a high incidence of melanoma and vitiligo-like skin depigmentation [7].

Since the Pura Raza Español (PRE) is a mainly grey breed, the animals are susceptible to vitiligo and melanoma. Since 2012, it has been standard practice to record a vitiligo score (VS) and melanoma score (MS) in PRE breed registers [8]. This provides the opportunity to estimate the breed prevalence of these disorders, and to examine potential environmental risk and genetic factors associated with these diseases. We hypothesised is that both VS and MS are prevalent in PRE populations worldwide, and that both environmental risk factors and genetics play a significant role in the development of these conditions. The specific aims of this work were to: (1) measure the breed prevalence of vitiligo and melanoma in a worldwide population of PRE, including non-grey horses, (2) establish the potential links with inbreeding, age, sex, coat colour and geographic area for the development of vitiligo and melanoma in this breed and (3) estimate the heritability and genetic correlations for VS and MS. A better understanding of how these factors contribute to the development of vitiligo and melanoma is crucial to improve the management and breeding selection of mainly grey horse breeds.

Material and methods

Population

96 The database included records of 11,436 animals (4,132 stallions and 7,304 mares) with a mean age
97 of 4.51 (s.d. 1.675) years (3–21 years old). These records were taken during the morphological
98 evaluation (between 2012 and 2016 from 36 countries) that all the PRE have once in their life
99 before being registered in the PRE-studbook [9]. Breeders are free to apply for this evaluation at
100 any time in the horse's life, and so animals of different ages are represented in the dataset. The
101 pedigree information (four complete generations, comprising 35,430 horses)was collected from the
102 official PRE-studbook.

103 *Outcome variables: vitiligo and melanoma phenotype*

104 The inspections were carried out by 18 previously-trained veterinarians who travel around the world
105 assessing horses for registration in the studbook. The VS was graded on a scale from 0 to 3 based
106 on inspection of the face [7] (**Supplementary Table 1)**. VS was not scored for the perianal and anal
107 regions. Although the appraisers took three different face scores for vitiligo for eyes, mouth and
108 nostrils, the genetic correlation between the nostril and mouth scores was close to 1, so they were
109 included as one single trait (nostril and mouth vitiligo score; NVS) evaluated together, and eye
110 vitiligo score (EVS) separately. The degree of vitiligo was scored in the same test as the MS [3],
111 which was detected by inspection and palpation. For the MS, a modified classification system on a
112 scale from 0 to 5 was used, as described in [10] **Supplementary Table 1**.

113 *Exposure variables*

114 The potential environmental risk factors studied were: age, sex, coat colour and geographic area of
115 the stud (the region where the horse was located when it was examined for vitiligo and melanoma).
116 The number of horses examined for each potential environmental risk factor by populations is
117 shown in **Supplementary Table 2**. An association frequency analysis of all the environmental risk
118 factors (M-L Chi-square) was also carried out (**Supplementary Table 3**). In addition, the degree of
119 inbreeding, defined as the probability that an individual possesses two identical alleles by descent at
120 a randomly chosen locus [11], was also studied (**Supplementary Table 4**).

121 *Descriptive statistics*

All the analyses were performed after dividing the database into 3 datasets: subpopulation 1 (S1; grey PRE: horses with three generations of ancestors with a grey coat colour (n=1719); subpopulation 2 (S2; grey PRE: horses with at least one generation of non-grey ancestors in the last three generations (n=3325)); subpopulation 3 (S3; non-grey PRE: horses with non-grey coat colour in any of the last three generations of ancestors (n=6392)).

The Kolmogorov–Smirnov normality test was used to verify whether the outcomes conformed or not to normal distributions. Next, a multivariate Generalized Non-linear Model (GLZ) was used to examine associations between the potential environmental risk factors, the interactions and the outcomes. The implemented GLZ method, is a robust general linear model which applies an ordinal multinomial distribution through a generalized logit function instead of the usual normal distribution. It also uses maximum likelihood methods in the generalized model to test whether a reduced model provides the same fit as a full model. The goodness of fit criteria of the models (Akaike Information Criterion and Bayesian Information Criterion) was also estimated. Additionally the maximum likelihood chi-square test was performed to study relationships among the environmental risk factors **(Supplementary Table 3)**. A simple linear regression analysis was carried out to describe the relationship between the outcomes and the inbreeding (**Supplementary Table 4**). Inbreeding was computed using Endog v4.8 software [12]. The other analyses were performed using the Statistica package for Windows, v.12.0 [13].

Genetic model

Heritability coefficients, genetic correlations and estimated breeding values (EBV) were estimated using restricted maximum likelihood (REML) methodology based on a multiple-trait animal model which was resolved using a Bayesian approach with TM software [14]. The outcomes were used in their linear scale (1 - 4 for EVS and NVS and 1 - 6 for MS, because TM is programmed to take 0 as a missing value). The Bayesian approach allowed us to work with non-normal traits. The equation in matrix notation to solve the mixed model was:

$y= Xb+Zu +e$, with:

$$\begin{bmatrix} \mathbf{u} \\ \mathbf{e} \end{bmatrix} \sim N\left(\begin{bmatrix} \mathbf{0} \\ \mathbf{0} \end{bmatrix}, \begin{bmatrix} \mathbf{A}\sigma_u^2 & \mathbf{0} \\ \mathbf{0} & \mathbf{I}\sigma_e^2 \end{bmatrix}\right)$$

where $\boldsymbol{y}$ is the vector of observations, $\boldsymbol{X}$ the incidence matrix of systematic effects, $\boldsymbol{Z}$ the incidence matrix of animal genetic effects, $\boldsymbol{b}$ the vector of systematic effects, $\boldsymbol{u}$ the vector of direct animal genetic effects, $\boldsymbol{e}$ the vector of residuals, σ_u^2 the direct genetic variance, σ_e^2 the residual variance, $\boldsymbol{I}$ an identity matrix and $\boldsymbol{A}$ the numerator relationship matrix.

The fitted model included only the environmental risk factors which were statistically significant for each outcome (Supplementary Table 2). The model included inbreeding as a covariate and the following systematic effects for each specific trait: age (≤3; 4-6 and >6); sex (male or female); geographic area (Spain, Europe, United States of America (USA) and Latin America), and coat colour (grey, bay, black and chestnut). The Bayesian approach was carried out four times separately for each population in a multivariate analysis with the three outcomes together.

Results

The breed prevalence of EVS, NVS and MS in the whole population was 2.8% (CI: 2.50%-3.10%), 20.5% (CI: 19.76%-21.24%) and 1.6% (CI: 1.37%-1.83%), respectively. In S1, the prevalence was 7.2% (CI: 5.98%-8.32%), 49.8% (CI: 47.44%-52.16%) and 4.0% (CI: 3.07%-4.92%); in S2, it was 3.6% (CI: 2.96%-4.93%), 31.6% (CI: 30.03%-33.18%) and 2.0% (CI: 1.52%-2.48%); and in S3, it was 1.2% (CI: 0.93%-1.46%), 6.9% (CI: 6.27%-7.52%) and 0.8% (CI: 0.06%-1.01%) for EVS, NVS and MS.

The prevalence of vitiligo and melanoma, the number of Pura Raza Español horses affected by each phenotypic score divided by the environmental effects, the p-value (including 2-way interactions), and the goodness of fit criteria (AIC and BIC) of the model are given in Table 1, Supplementary Table 2 and Supplementary Figure 1. Sex was significantly associated with EVS (except in S3) and NVS. Most of the interactions sex with age/geographic area/coat colour were not significant although some were significant (the most important sex-age in S1 for NVS). Age was significantly associated with EVS, NVS and MS for all the coat colours and S1 and S2, and the interaction age-

coat colour was relevant in S1. For non-grey PRE, age was not a significant risk factor for EVS and MS (only the interaction age coat colour was significant for EVS).

Coat colour was significantly associated with all the traits in the whole population (55.54% of the entire current PRE pedigree have a grey coat). In fact, for vitiligo, the vast majority were grey (465 of 597 young horses studied with an NVS greater than 1). When only non-grey PRE were considered, coat colour was not significantly associated with MS. Although the interaction age-coat colour was significant for the three traits in the whole population, in 3 year-olds, the coat was not an important factor: only 35 of the 3728 PRE of this age had a MS greater than 1, and of these 35, 18 did not have a grey coat (12 were bay, 7 black and 1 chestnut). The geographic area was significantly associated with EVS, NVS and MS. Inbreeding was a significant risk factor in the whole population and particularly for NVS in the grey PRE population.

Heritability coefficients and genetic correlations of all traits in the four scenarios considered in the study are shown in Table 1. Heritability ranged from 0.88 (s.d. +0.057) for MS in S1 to 0.07 (s.d. +0.018) for EVS in S2 and 0.07 (s.d. +0.022) for MS in the S3. The genetic correlation ranged from 0.09 (s.d. +0.202) for EVS-MS in S3 to 0. 57 (s.d. +0.127) for EVS-MS in S1. The EBV for the three traits studied, classified according to phenotypic class, is represented in Figure 1. The EBV ranged from 0.37 (s.d. +0.091) to -0.09 (s.d. +0.085) for EVS, 1.59 (s.d. +0.305) to -0.64 (s.d. +0.328) for NVS and 0.60 (s.d. +0.189) to -0.07 (s.d. +0.072) for MS.

Discussion

This study has, for the first time, addressed a breed prevalence of vitiligo and melanoma using a large worldwide population of PRE. Our results showed that mouth and nostril vitiligo are prevalent in PRE: nearly 21% of the PRE studied of any sex, age, coat colour and worldwide area, were affected. Eye vitiligo and Melanoma had lower prevalences. However, the prevalence of all these defects was higher in grey horses with three generations of grey ancestors (S1) and lower in non-grey horses with non-grey ancestors in any of the last three generations (S3). There were differences

198 in the prevalence of EVS between all the subpopulations examined, and the prevalence for the
199 disorders did not overlap among the subpopulations. The difference in the case of non-grey horses
200 can be explained by the assumed non-existence of the 4.6-kb duplication in intron 6 of STX17. In
201 addition, the differences found between the two grey populations can be explained by the assumed
202 higher frequency of the 4.6-kb duplication in intron 6 of STX17 of S1 horses, since their
203 predecessors were also grey and the STX17 gene is epistatic towards all other coat colour genes,
204 except the KIT gene that conditions white-coloured coats [15]. These prevalence results were in
205 concordance with previous studies and confirm that both vitiligo and melanoma are associated with
206 the grey coat colour [7,16–19]. Similarly, a relationship between grey coat colour and equine
207 melanoma has long been recognized [3,4]. In particular, a fully dominant and autosomal trait
208 controlled by a cis-acting regulatory mutation, more specifically a 4.6 kb duplication in intron 6 of
209 syntaxin-17, has been proposed as the probable genetic link between grey coat colour and
210 melanoma [18]. This mutation is associated with 4 genes: NR4A3 (nuclear receptor subfamily 4,
211 group A, member 3), STX17 (syntaxin 17), TXNDC4 (thioredoxin domain-contain-4') and INVS
212 (inversin), with notably higher levels of STX17 and NR4A3 present in melanomas [7,18,20].
213 Horses that were homozygous for the mutation turn grey more rapidly and are also more uniformly
214 white with less speckling than heterozygous horses. Homozygotes were also found to exhibit a
215 significantly higher incidence of melanoma [18].
216 The prevalence of any degree of melanoma was 1.6% (CI: 1.37%-1.83%) in the whole population.
217 Previous studies, with numbers of horses ranging between 58 [16] and 1,119 [7], have described a
218 very high prevalence in PRE (89.6%; only analyzed PRE with generalized progressive leukotrichia)
219 [16], followed by Lipizzaner (50%) [17], Camargue (31.4%) [21], Old Kladruber horses (12.5%)
220 [19] and Quarter horses (16%) [20].
221 Our results showed that the prevalence of EVS in chestnut horses was comparable (3.5%; IC: 2.1%-
222 4.9%) with grey horses. There are no previous studies that address the study of EVS in chestnut

223 horses, and these results could therefore point to the pleiotropic role of coat colour genes in the
224 development of horse these disorders, or simply indicate an absence of any relationship.
225 Our study also confirmed the effect of age and coat colour (the interaction age/coat colour had a p-
226 value < 0.001 in the whole population) on the prevalence of vitiligo and melanoma in horses, as
227 previously described [3,7,17,19,21]. Melanoma can also affect young animals of any coat colour
228 such as melanocytic naevi. However, these are rare, and in general mostly highly malignant
229 exceptions, with an aggressive neoplasm influenced by different genetic mechanisms. This suggests
230 the existence of a different biological pathway in the melanoma development at early ages not
231 linked to coloration, distinct from the mostly benign condition in grey horses linked with the age
232 and greying regulation (e.g. *dermal melanomatosis*) [17,22,23]. In fact, the interaction age/coat
233 color was not only significant in the S3 population for Melanoma. Many of the horses in this study
234 were sampled young, but our study included animals across all age categories. This has allowed us
235 to study the prevalence in a world population of young animals, whereas most previous studies have
236 focused on adult horses and have included large populations. However, given the links between
237 grey coat colour, melanoma and age, it seems necessary to continue analyzing this morphologic
238 evaluation as the PRE horses grow older.
239 As previously published [19,24], sex and the interactions of sex with age/coat color and
240 geographical area were not significantly associated with melanoma (except the interaction sex and
241 geographical area in S2). In the Camargue breed, a higher prevalence of melanoma was found in
242 geldings compared with mares and stallions, but the differences were not statistically significant
243 [21]. Sex and its interaction with coat colour were significantly associated with VS except for EVS
244 in S3, which suggests that differential biological pathways may be involved in these disorders.
245 In this study, inbreeding was significant associated with all the outcomes studied in the whole
246 population. Although this result was consistent with previous findings on melanoma in the Old
247 Kladruber population [25], it was not apparent in the subpopulation analyses. Regression
248 coefficients had a numerically greater effect on vitiligo than on the MS. Although the effect of

inbreeding appeared to be statistically significant, low or low-moderate values of regression coefficients were found, which indicates that there were minimal or no changes in the melanoma and VS as the inbreeding increased [19]. In fact, NVS, EVS and MS outcome scores increased by only 0.03, 0.18 and 0.01, respectively, for each 10% increase in inbreeding. Due to this, although the statistically-significant values of the inbreeding effect indicate the possibility of inbreeding depression, the influence from a practical point of view on the outcomes studied is minimal, as has been observed in the Old Kladruber population [25].

Geographic area was significantly associated with all traits, although clear reasons as to how this affected melanoma and vitiligo are not clear. It is accepted that Melanoma in horses is not affected by exposure to the sun, and in view of its frequent occurrence in shaded areas of the skin, sun exposure is not a primary risk factor in these horses [21]. Geographic area had interactions with other environmental factors as the coat colour in S1 and S3, since significant differences were found in the distribution of the geographic area with coat colour and age (**Supplementary Table 3**), but the influence of the geographic area is not concordant with a higher percentage of grey animals, since it ranges from 42.6% for USA to 53.8% in Latin America, while the geographic areas with higher prevalence values in the whole population studied were USA for EV and MS and Latin America for NVS. It is also true that there is a complex relationship between geographic areas, coat colour, age and sex (**Supplementary Table 3**), and so, while there might be environmental and perhaps management effects associated with particular geographic areas, colour and age, these interactions cannot be ruled out as reasons or confounders to these effects. So, in future studies, these 2-way (and even 3-way) environmental interaction effects should be addressed to get a better understanding of the non-genetic effects that may influence vitiligo and melanoma disorders. There is a hypothetical veterinarian effect in these traits, however the same Spanish veterinarians travel around the world to examine the entire PRE population. Nevertheless the veterinarian examining each horse was not recorded, which is a limitation of this study. Future studies on a new linear

scoring system, which has great potential for breeding programs will also address a potential observer effect.

This is the first time that over 10,000 horses have been analyzed. Despite the fact that heritability for the grey population was within the range of previous studies for MVS and MS outcomes [7,17,19], it was out of range for non-grey horses. This suggests the most severe forms of these diseases have less heritability, with an increased role of environmental effects, which is not the case in their most benign forms [17,22,26,27]. This is also the first time that the heritabilities have been estimated in a population with various coat colours. Although our study agrees with the theory that vitiligo and melanoma are strongly influenced by the grey coat [17,22,26,27], we found clear differences for melanoma in the environmental risk factors, prevalence and genetic parameters amongst non-grey horses. Our results agree with a previous study [7] and suggest that the estimated genetic correlation between melanoma and vitiligo was negligible. In fact, in the non-grey population there were no significant genetic correlations between melanoma and vitiligo. Such a low genetic correlation, together with the different heritability value between EVS and NVS, reaffirms the idea that these traits are regulated by different mechanisms. Nevertheless, the genetic correlation between VS and EVS ranged from 0.21 to 0.48, which leads us to believe that selective breeding to avoid one vitiligo trait may effectively reduce both traits in a population. The EBV were in concordance with the phenotypic score for melanoma and vitiligo, and they could therefore be used to decrease their prevalence in the PRE grey population, and perhaps in other mainly grey horse breeds. The EBV for NVS had a wider range, and except in grey horses with grey ancestors, the highest heritability, and should therefore be the most effective trait to use in a breeding program, as well as being used to avoid EVS, due to its medium-level genetic correlation. MS had a relatively narrow range of EBV, but extremely high heritability in S1, and so the selection of PRE with lower EBV for MS should be effective in breeding to reduce prevalence in this subpopulation.

The added genetic predisposition to vitiligo and melanoma in PRE, with a low-moderate heritability rate for the population as a whole and a high heritability rate for the grey population, indicates that

the prevalence of these defects could be effectively reduced by genetic selection, mainly in the grey population, by choosing grey PRE with lower EBV for reproduction. The development of vitiligo and melanoma is associated with environmental risk factors such as inbreeding, age sex, coat colour and geographic area. Although these results should empower breeders to improve the health of PRE, further studies are still needed to better understand the genetic and environmental factors associated with vitiligo in this and other horse breeds. Nonetheless, this study may help initiate a genetic selection programme to considerably decrease their prevalence in PRE.

FIGURE 1: Estimated breeding values for vitiligo and melanoma scores in a population of 11,436 Pura Raza Español horses.

311 **TABLES**

312 **TABLE 1.** Prevalence (%) for vitiligo and melanoma scores in the populations of Pura Raza
313 Español horses and results of the generalized non-linear models analysis for each investigated
314 environmental effect

315

Environmental effects		Whole population			Subpopulation 1			Subpopulation 2			Subpopulation 3		
		EVS [1]	NVS[2]	MS[3]	EVS	NVS	MS	EVS	NVS	MS	EVS	NVS	MS
Sex	Male	4.02	24.13	1.65	10.69	57.10	4.69	5.51	37.49	1.45	1.37	8.22	0.86
	Female	2.11	18.41	1.57	4.83	44.98	3.47	2.71	28.45	2.25	1.09	6.12	0.72
	p	<0.001	<0.001	0.7	<0.001	<0.001	0.2	<0.001	<0.001	0.1	0.3	0.001	0.5
Age	<4	2.36	16.01	0.94	5.21	40.41	0.74	2.74	24.24	1.27	1.48	6.09	0.83
	4-6	2.69	21.37	1.27	6.57	52.17	3.18	3.82	33.19	1.33	1.08	7.13	0.75
	>6	4.63	29.07	5.18	13.81	61.51	14.23	5.05	41.35	6.73	0.81	8.25	0.65
	p	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.03	<0.001	<0.001	0.3	0.05	>0.9
Coat colour	Grey	4.84	37.71	2.66	-	-	-	-	-	-	-	-	-
	Bay	0.79	5.99	0.72	-	-	-	-	-	-	0.79	5.99	0.72
	Black	1.29	4.60	0.91	-	-	-	-	-	-	1.29	4.60	0.91
	Chestnut	3.51	17.99	0.76	-	-	-	-	-	-	3.51	17.99	0.76
	p	<0.001	<0.001	<0.001	-	-	-	-	-	-	<0.001	<0.001	0.7
Geographic area	EEUU	3.68	21.15	3.59	6.38	38.30	7.09	4.19	34.19	6.77	2.80	10.53	1.15
	Europe	3.22	18.37	2.65	5.48	43.84	4.11	5.03	23.90	3.14	1.69	9.12	2.03
	Latinamerica	2.70	22.01	1.08	6.77	49.62	3.76	3.58	28.81	0.75	0.44	5.99	0.29
	Spain	2.68	20.26	1.37	7.43	51.49	3.63	3.48	32.22	1.55	1.06	6.41	0.71
	p	0.3	0.3	<0.001	0.8	0.01	0.5	0.7	0.5	<0.001	0.003	0.002	0.04

316 [1]Eye vitiligo score [2]Nostril and mouth vitiligo score [3]Melanoma Score
317 Subpopulation 1 (PRE greys with the three preceding generations with grey coat colour), 2 (PRE
318 greys with at least one of the three preceding generations with non-grey coat colour) and
319 Subpopulation 3 (Non- grey PRE with the three preceding generations with non-grey coat colour)
320

321

TABLE 2: Heritability coefficients on diagonal and genetic correlations on off-diagonal with standard deviations among the vitiligo and melanoma scores in the four Pura Raza Español subpopulations studied

	EVS[1]	NVS[2]	MS[3]
Whole population			
EVS	0.09 (0.019)	0.42 (0.084)	0.21 (0.156)
NVS		0.44 (0.031)	0.15 (0.096)
MS			0.13 (0.037)
Subpopulation 1			
EVS	0.61 (0.111)	0.51 (0.126)	0.57 (0.127)
NVS		0.62 (0.072)	0.27 (0.085)
MS			0.88 (0.057)
Subpopulation 2			
EVS	0.07 (0.018)	0.33 (0.164)	-0.34 (0.334)
NVS		0.47 (0.056)	0.10 (0.187)
MS			0.16 (0.058)
Subpopulation 3			
EVS	0.09 (0.017)	0.18 (0.142)	0.09(0.202)
NVS		0.15 (0.030)	-0.10 (0.170)
MS			0.07 (0.022)

[1]Eye vitiligo score

[2] Nostril and mouth vitiligo score

[3] Melanoma score

Subpopulation 1 (PRE greys with the three preceding generations with grey coat colour), 2 (PRE greys with at least one of the three preceding generations with non-grey coat colour) and Subpopulation 3 (Non- grey PRE with the three preceding generations with non-grey coat colour)

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