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Regressive Evolution in the Mexican Cave Tetra, *Astyanax mexicanus*

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Summary

Cave adapted animals generally have reduced pigmentation and eyes, but the evolutionary forces driving the reductions are unknown; Darwin famously questioned the role of natural selection in eye loss in cave fishes; “As it is difficult to imagine that eyes, although useless, could be in any way injurious to animals living in darkness, I attribute their loss wholly to disuse” [1]. We studied the genetic basis of this phenomenon in the Mexican cave tetra, *Astyanax mexicanus*, by mapping the quantitative trait loci (QTL) determining differences in eye/lens sizes and melanophore number between cave and surface fish. In addition, we mapped QTL for the putatively constructive traits of jaw size, tooth number, and numbers of taste buds. The data suggest that eyes and pigmentation regressed through different mechanisms. Cave alleles at each eye/lens QTL we detected caused size reductions. This uniform negative polarity is consistent with evolution by natural selection and inconsistent with evolution by drift. In contrast, QTL polarities for melanophore number were mixed, consistent with evolution by genetic drift or indirect selection through pleiotropy. Past arguments against a role for selection in regression of cave fish eyes cited the insignificant cost of their development [2,3], but we argue that the energetic cost of their maintenance is sufficiently high for eyes to be detrimental in the cave environment. Regression, a ubiquitous aspect of all evolutionary change, can be caused either by selection or genetic drift/pleiotropy.

Results and Discussion

Absence of light drives the evolution of cave animals towards a suite of characteristic, cave-related (troglomorphic) phenotypes. In the dark, eyes and pigmentation lose their functions, and tend over the generations to regress or disappear. Without light there is no photosynthesis, and the trophic base of many cave communities is narrow. Cave animals typically cope with the scarcity of food by evolving more sensitive tactile and chemical senses and slower or more efficient metabolisms. Compensatory changes like these probably evolve because of strong selection, but what causes the regression of eyes and pigmentation? The three modern competing hypotheses for eye regression are natural selection, recurrent mutation/genetic drift, and pleiotropy [2].

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Astyanax mexicanus is an ideal species to study the genetics of troglomorphy because it has both eyed surface and cave adapted populations, all of which are interfertile. Cave fish were collected from Pachón cave in NE Mexico [locality map in 4] and surface fish were collected from nearby streams (Supplemental Fig. 1). We hybridized Pachón cave and surface fish, creating a mapping progeny of 539 F_2 siblings. We mapped 178 loci in the cross (2191 cM) for an average distance between adjacent markers of 14.7 cM. We phenotyped the F_2 fish by measuring eye size, lens size, counting the density of melanophores in four places on the bodies, measuring the lengths of the dentary and maxillary bones in the jaw apparatus, and counting maxillary teeth and taste buds (Table 1 lists sample sizes for the different traits). This gave us a set of standardized phenotypes that could be correlated with genotypes. Phenotypic and genotypic data (see Supplemental Online Material) were used to identify chromosome regions where genes affecting the traits were located. Quantitative trait loci (QTL) were detected in two phases, first by simple interval mapping (SIM) of putative QTL, followed by a refinement phase using multiple interval mapping algorithms (MIM). We used MultiQTL software (www.multiqtl.com), with $P < 0.05$ and a false detection rate < 0.10. (Supplemental Online Material details Material and Methods.)

With few exceptions, phenotypic correlations among traits in the F_2 are weak or non-existent (Table 1). Not all correlations could be determined because some traits (notably lens sizes) were assessed in different siblings but, of the 26 correlations we calculated, only six were significant at the $P = 0.01$ level, and three others at the $P = 0.05$ level. Eye size was significantly negatively correlated with three melanophore traits and the number of maxillary teeth and positively correlated with lens size. MelE and MelD were strongly correlated, and the length of the maxillary bone was significantly correlated with the length of the dentary and the number of taste buds. It is notable that eye size was not significantly correlated with the lengths of the dentary or maxillary or number of taste buds.

We detected 48 QTL for these traits: eight affecting eye size, six affecting lens size, 18 affecting pigmentation, seven affecting lengths of the jaw bones, six affecting the number of maxillary teeth, and three affecting the number of taste buds (Table 2). The total proportions of variance explained by QTL for each trait ranged from 0.11 to 0.77 (mean = 0.44) and the total proportions of additive variance explained ranged from 0.03 to 0.52 (mean = 0.28).

Some of the QTL co-mapped and might represent the effects of single genes or tightly clustered genes (Supplemental Fig. 2). On LgP7, LgP9, and LgP15, there were two, four and two co-mapping QTL for different melanophore traits. On LgP8 and LgP20, QTL for eye and lens size co-map. Because of the possibility that these sets of co-mapping QTL each represent single loci, for statistical comparisons of eye and melanophore QTL we counted each region only once. On LgP13 QTL for maxillary size and number of maxillary teeth co-map. While these may represent one gene, the polarities of substitution effects are in disagreement, with smaller maxillae associated with more teeth. On LgP27, QTL for eye size and number of maxillary teeth co-map. On LgP5 and LgP25, QTL for eye or lens size co-map with QTL for taste-buds. Other examples of co-mapping traits can be seen in Supplemental Figure 2.

Trait means (μ), and estimates of allelic substitution (d) and heterozygous effects (h) are given in Table 2. Expected trait values for cave and surface homozygotes and heterozygotes are $\mu + d$, $\mu - d$, and $\mu + h$, respectively. We calculated trait values for all 48 QTL and identified loci at which the heterozygote fell between the two homozygotes as intermediate in dominance. Based on this criterion, 36 of the cave alleles are of intermediate dominance. The remaining 12 loci cannot be classified unambiguously because the standard errors of estimate sometimes exceeded the differences in trait values among genotypes, but at four of the loci the cave allele seems recessive, at two it seems dominant, and at two more it seems clearly overdominant. We

calculated a measure of dominance as the absolute value of the ratio of h/d and found the median value to be 0.44, or semi-dominant.

In order to compare patterns of substitution between eye/lens and melanophore QTL, the two regressive trait classes, we calculated trait values for all three genotypes at each QTL using estimates of d, h, and μ. To standardize the scales, we divided expected trait values by their trait means. In the three cases in which two or more melanophore QTL co-mapped and it was possible that single genes were affecting multiple traits, the scaled trait values were averaged for each genotypic class. This reduced the number of melanophore QTL to 13 for statistical testing. In the two cases where eye and lens QTL co-mapped, we chose the one with the higher LOD score to represent the QTL.

The patterns of substitution effects differ radically between QTL for eye/lens size and melanophore numbers. Cave alleles at all 12 eye/lens QTL effect relatively modest, but steady decreases of eye/lens size (Fig. 1a). In contrast, cave alleles at QTL affecting melanophore number have positive (n = 5), as well as negative slopes (n = 8), and their substitution affects are much larger (Fig. 1b). The distributions of polarities differ significantly between the two classes of traits (12:0 *vs*. 8:5, 2-tail P = 0.039, Fisher's exact test). Comparison of the slopes for the two trait classes (Fig. 1) also reveals an obvious difference in dispersion (Wilcoxon two-sample statistic for testing homogeneity of variances, $R_{11,13} = 186$, P = 0.005).

Our interpretation of these differences in effects between the two classes is that regression of eyes came about primarily through selection, while decreases in numbers of melanophores resulted mainly from recurrent mutation/genetic drift or indirectly through pleiotropy. If there were strong direct selection against melanophores, it is unlikely that five QTL, all with major effects, would have cave alleles increasing the numbers of melanophores. If eye/lens reduction were accomplished through genetic drift, it is unlikely that the pattern of effects would contrast so radically with that for melanophores.

If eyes regressed through selection, was the selection directed against the eye itself or was it indirect, through negative pleiotropy of alleles selected for affects on other traits? Hedgehog signaling pathways direct the development of midline structures, including jaws, teeth and tastebuds (reviewed in 5). Hedgehog activities also have important affects on eye development, in part, because *Hh* expression is antagonistic to that of *PAX6* and alters patterns of expression of *PAX2*. Yamamoto *et al.* [5] have shown through experimental alteration of gene activity in *A. mexicanus* embryos that hedgehog activity is a strong determinant of eye size. Increased unilateral expression of *sonic hedgehog (shh)* and *tiggy-winkle hedgehog* (*twhh*) in surface fish suppresses the development of the treated eye. Thus, one hypothesis is that increased feeding efficiency may be an important adaptation in cave fish, accomplished through up-regulation of hedgehog signaling but at the expense of eye development [6].

The *Hh* hypothesis has two parts. The first is that up-regulation of hedgehog activity suppresses development of the eyes; the second is that hedgehog activity was up-regulated during cave fish evolution by selection to improve feeding efficiency and that this was the primary cause of eye regression. The evidence linking hedgehog activity to eye development seems compelling, but our data do not yet provide a definitive test of the second part of the hypothesis, although they suggest it cannot be the sole explanation of eye regression. Six QTL for eye/lens size co-map with QTL affecting feeding traits (jaw bone sizes, numbers of teeth and taste-buds), but six others do not, and the QTL in the latter group control a much greater proportion of explained additive variance than those in the former (not co-map *vs*. co-map groups: Eye: 0.233 *vs*. 0.087; LensE: 0.364 *vs*. 0.070; LensL: 0.014 *vs* 0.015). Furthermore, it is not just feeding trait QTL and eye/lens QTL that co-map. Feeding trait QTL co-map with QTL for melanophore numbers three times and QTL for eye/lens size and melanophore number co-map

four times. We attribute this co-mapping to a general tendency towards pleiotropy with these traits [7] rather than to any specific relationship between feeding efficiency and eye loss. In addition, if the QTL affecting feeding traits were major contributors to eye regression, we might expect to see strong negative phenotypic correlations between these traits and eye size in the F_2. Such correlations are weak or non-existent (Table 1). In sum, definitive tests of the generality of the second part of the Hh hypothesis await the molecular identification of the genes underlying eye loss and feeding morphology, and characterization of the fitness effects of their alleles.

We also mapped candidate genes *shh* (LgP28), *twhh* (LgP15) and *PAX6* (LgP10). No eye QTL are located near these loci, making it unlikely that mutations in any of them are directly responsible for eye regression. One eye QTL maps to a point near the gene for ocular and cutaneous albinism (*OCA2*, LgP5).

Is it possible that Darwin's premise was simply incorrect? Are eyes in a cave disadvantageous, and if so, why? In essence, the argument against selection is that the cost of making an eye is trivial compared to the cost of its replacement tissue in the socket [2,3], or that the developmental cost is paid by cave fish anyway because the eyes start developing and only degenerate after many cell cycles of tissue growth and replacement [4]. However, modern physiology and molecular biology suggest these arguments might address the wrong costs. The vertebrate retina is one of the most energetically expensive tissues, with a metabolism surpassing even that of the brain [8]. Underscoring this high metabolic demand is the observation that one manifestation of genetic defects decreasing the efficiency of mitochondria is blindness (*e.g.*, Leber's Hereditary Optical Neuropathy [9]). Thus, maintenance of eyes might pose a significant burden in the cave environment. Increasing this burden, the vertebrate retina uses more energy in the dark than in the light, because the membranes of the photoreceptor disks must be maintained in the hyperpolarized state until depolarized in response to light [10,11]. Oxygen consumption by the vertebrate retina is approximately 50% higher in the dark than in the light [8]. Adding further to the retina's cost is its structural maintenance. Ten percent of the photoreceptor outer disks in vertebrates are shed and renewed each day, and the structure may be completely replaced over 35 times yearly [12].

Thus, while the energetic cost of making an eye may be trivial, the expense of maintaining one is much greater. In the dark, it may be costly enough to create effective selection for eye regression. In contrast, the argument of metabolic cost cannot be made for regression of pigmentation, and the QTL trait value data (Fig. 1) show that the two traits have regressed through different mechanisms.

This study shows that regression may be effected by active selection as well as by the passive accumulation and fixation of damaging mutations, and that the various possibilities can be distinguished by the patterns of allelic substitutions involved. Thus, regression, an integral part of the progress of evolutionary change, can be accomplished in a variety of ways.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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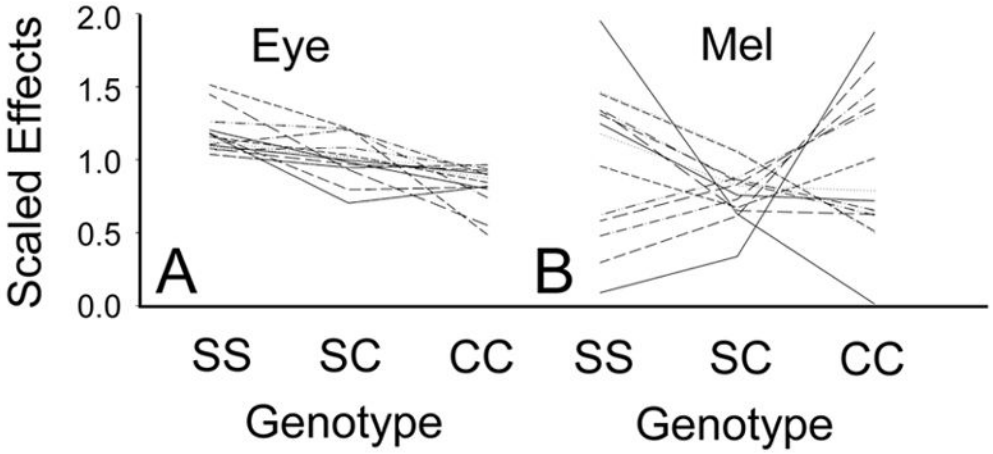

Figure 1.
Standardized trait values for surface homozygotes (SS), heterozygotes (SC) and cave homozygotes (CC), for eye/lens and Melanophore QTL.

Table 1

Phenotypic correlations among the traits assessed in this study. Correlation coefficients above the diagonal and case-wise sample sizes below the diagonal. The six correlations significant at $P \leq 0.01$ are shown in italicized bold face and the three significant at $P \leq 0.05$ are shown in bold. N is the number of individuals typed for each trait.

Trait	N	Eye	MelD	MelE	MelA	MelL	MaxTth	TBuds	Dentary	Maxillary	LensE	LensL
Eye	539	------	**−0.170**	−0.050	**−0.184**	***−0.231***	**−0.148**	−0.173	−0.025	−0.037	0.039	***0.709***
MelD	174	174	------	***0.750***	MD	MD	−0.022	0.033	0.047	−0.136	MD	MD
MelE	174	174	174	------	MD	MD	0.043	0.091	0.123	−0.009	MD	MD
MelA	128	128	MD	MD	------	0.145	MD	MD	MD	MD	MD	MD
MelL	128	128	MD	MD	128	------	MD	MD	MD	MD	MD	MD
MaxTth	227	227	168	168	MD	MD	------	0.095	0.060	0.042	MD	MD
TBuds	117	117	77	77	MD	MD	114	------	0.106	***0.247***	MD	MD
Dentary	218	218	162	162	MD	MD	213	108	------	***0.344***	MD	MD
Maxillary	219	219	162	162	MD	MD	215	110	217	------	MD	MD
LensE	115	115	MD	MD	MD	MD	MD	MD	MD	MD	------	***0.479***
LensL	112	112	MD	MD	MD	MD	MD	MD	MD	MD	112	------

Table 2

QTL for cave related traits detected in the analysis of a cross between Pachon cave and surface *Astyanax mexicanus*. Trait and trait means are listed in column 1. LOD scores and P values for SIM and MIM analyses are listed separately. Also listed are explained proportions of total and additive variance (PEV, PEVad), and substitution (d) and heterozygous (h) effects based on MIM analysis. Expected trait values for cave and surface homozygotes and heterozygotes are μ + d, μ − d, and μ + h, respectively. The QTL are mapped in Supplemental Figure 1.

Trait Mean (μ)	Linkage Group	SIM LOD	P value	MIM LOD	P value	QTL Position (cM)	PEV	PEVad	Subst.effect (d)	Heter.effect (h)
Eye Size 0.92	LgP4	3.22	0.004	4.19	0.002	38.3 ± 13.7	0.031 ± 0.013	0.029 ± 0.012	−0.091 ± 0.021	−0.004 ± 0.019
	LgP5	5.29	0.001	5.01	0.002	64.8 ± 10.6	0.031 ± 0.011	0.023 ± 0.010	−0.081 ± 0.020	0.019 ± 0.029
	LgP7	16.00	0.001	10.99	0.002	65.9 ± 33.0	0.080 ± 0.021	0.072 ± 0.018	−0.145 ± 0.020	0.027 ± 0.022
	LgP8	14.73	0.001	3.37	0.002	32.4 ± 22.5	0.066 ± 0.041	0.013 ± 0.009	−0.060 ± 0.021	0.076 ± 0.051
	LgP11	1.78	0.100	3.07	0.006	67.2 ± 23.2	0.027 ± 0.013	0.018 ± 0.009	−0.071 ± 0.020	−0.030 ± 0.024
	LgP19	2.00	0.100	2.81	0.004	48.7 ± 9.4	0.025 ± 0.012	0.005 ± 0.004	−0.034 ± 0.019	−0.052 ± 0.020
	LgP20	22.50	0.001	20.15	0.002	65.6 ± 0.1	0.117 ± 0.020	0.114 ± 0.020	−0.186 ± 0.018	−0.017 ± 0.013
	LgP27	11.10	0.001	8.37	0.002	1.3 ± 2.2	0.048 ± 0.014	0.046 ± 0.014	−0.117 ± 0.019	0.010 ± 0.013
LensL 1.00	LgP8	4.11	0.040	7.24	0.001	13.3 ± 15.8	0.293 ± 0.081	0.204 ± 0.065	−0.513 ± 0.108	0.214 ± 0.127
	LgP14	4.83	0.010	8.18	0.001	103.2 ± 4.9	0.131 ± 0.029	0.057 ± 0.034	−0.260 ± 0.084	0.210 ± 0.061
	LgP20	5.09	0.010	8.64	0.001	65.3 ± 1.0	0.171 ± 0.037	0.160 ± 0.041	−0.448 ± 0.067	−0.069 ± 0.046
	LgP25	1.58	0.060	3.85	0.002	3.1 ± 3.6	0.081 ± 0.030	0.013 ± 0.014	−0.104 ± 0.078	0.204 ± 0.048
LensE 1.00	LgP6	1.88	0.070	2.65	0.006	32.2 ± 8.1	0.043 ± 0.022	0.015 ± 0.016	−0.185 ± 0.125	−0.203 ± 0.073
	LgP15	2.43	0.010	3.54	0.002	35.4 ± 6.7	0.070 ± 0.026	0.014 ± 0.014	−0.180 ± 0.119	−0.296 ± 0.066
MelAnal 15.10	LgP7	2.41	0.055	7.69	0.001	37.3 ± 17.6	0.100 ± 0.034	0.064 ± 0.033	10.497 ± 3.164	−5.609 ± 1.472
	LgP9	2.94	0.027	15.22	0.001	24.0 ± 0.9	0.104 ± 0.019	0.063 ± 0.018	10.777 ± 1.673	−6.157 ± 1.007
	LgP14	5.80	0.002	7.62	0.001	40.2 ± 17.7	0.212 ± 0.066	0.104 ± 0.045	13.646 ± 3.673	−9.848 ± 2.185
	LgP15	4.00	0.001	10.54	0.001	38.2 ± 1.4	0.103 ± 0.025	0.096 ± 0.024	−13.125 ± 1.887	−2.273 ± 1.261
	LgP19	3.32	0.003	11.1	0.001	9.1 ± 4.5	0.157 ± 0.032	0.123 ± 0.027	−14.818 ± 1.908	−5.406 ± 1.271
	LgP23	2.36	0.022	5.44	0.001	2.2 ± 7.4	0.047 ± 0.016	0.018 ± 0.012	−5.433 ± 2.102	−5.047 ± 1.148
	LgP25	1.47	0.094	6.61	0.000	0.0 ± 0.7	0.051 ± 0.018	0.034 ± 0.014	7.697 ± 1.698	−3.880 ± 1.123
MelDorsal 18.40	LgP6	2.46	0.016	4.02	0.001	41.3 ± 4.4	0.079 ± 0.034	0.007 ± 0.010	0.497 ± 2.673	−5.786 ± 1.720
	LgP7	2.67	0.032	4.66	0.001	104.1 ± 31.6	0.114 ± 0.038	0.073 ± 0.033	8.252 ± 2.261	−3.704 ± 2.680
	LgP9	7.38	0.001	11.25	0.001	31.7 ± 6.5	0.218 ± 0.050	0.187 ± 0.040	−13.270 ± 1.833	−3.583 ± 1.470
	LgP15	2.45	0.020	3.14	0.005	36.7 ± 16.0	0.079 ± 0.034	0.008 ± 0.012	−1.986 ± 2.151	5.784 ± 1.589
	LgP25	2.62	0.004	4.19	0.001	9.9 ± 4.1	0.067 ± 0.026	0.028 ± 0.018	−4.932 ± 1.812	−4.204 ± 1.353
MelEyes 36.80	LgP7	2.02	0.092	6.01	0.001	117.1 ± 20.7	0.085 ± 0.027	0.070 ± 0.030	13.391 ± 3.475	−3.977 ± 2.339
	LgP9	10.46	0.001	16.1	0.001	31.6 ± 5.2	0.305 ± 0.039	0.301 ± 0.039	−28.103 ± 2.533	−1.478 ± 1.855
	LgP14	2.23	0.080	4.55	0.001	139.0 ± 17.1	0.059 ± 0.019	0.024 ± 0.017	−7.257 ± 3.438	−6.055 ± 3.333
	LgP26	2.77	0.014	4.31	0.001	63.2 ± 20.5	0.145 ± 0.066	0.124 ± 0.067	−17.514 ± 5.929	2.632 ± 4.599
MelLat 19.30	LgP9	4.09	0.002	6.09	0.001	45.1 ± 9.4	0.241 ± 0.062	0.222 ± 0.065	−13.325 ± 2.388	1.888 ± 2.022
	LgP18	1.75	0.057	2.5	0.011	4.5 ± 6.4	0.082 ± 0.046	0.058 ± 0.035	−6.424 ± 2.516	−2.344 ± 2.137
Dentary 1.00	LgP4	1.79	0.087	4.08	0.001	76.0 ± 10.6	0.069 ± 0.026	0.009 ± 0.010	−0.009 ± 0.007	0.021 ± 0.006
	LgP6	1.97	0.055	4.93	0.001	12.2 ± 5.4	0.104 ± 0.034	0.004 ± 0.006	−0.002 ± 0.008	−0.028 ± 0.006
	LgP10	2.14	0.059	5.36	0.001	26.3 ± 23.2	0.165 ± 0.062	0.129 ± 0.051	0.044 ± 0.011	0.014 ± 0.009
	LgP26	2.26	0.038	4.04	0.001	81.0 ± 12.2	0.111 ± 0.043	0.008 ± 0.012	0.006 ± 0.010	−0.028 ± 0.007
Maxillary 1.00	LgP2	1.76	0.034	2.56	0.005	9.1 ± 2.6	0.029 ± 0.014	0.009 ± 0.008	0.012 ± 0.006	−0.013 ± 0.005
	LgP11	2.62	0.018	3.44	0.005	97.6 ± 25.5	0.065 ± 0.026	0.054 ± 0.028	0.031 ± 0.011	−0.007 ± 0.008
	LgP13	2.81	0.004	4.22	0.001	9.7 ± 2.6	0.049 ± 0.019	0.040 ± 0.019	−0.027 ± 0.007	−0.008 ± 0.006
MaxTth 2.59	LgP6	4.51	0.001	6.38	0.001	25.3 ± 7.1	0.121 ± 0.037	0.060 ± 0.025	0.615 ± 0.146	−0.441 ± 0.104
	LgP13	5.99	0.001	4.97	0.005	14.6 ± 3.6	0.061 ± 0.019	0.042 ± 0.022	0.500 ± 0.143	0.196 ± 0.155
	LgP14	4.51	0.001	3.94	0.005	133.3 ± 38.9	0.065 ± 0.030	0.053 ± 0.025	0.561 ± 0.189	−0.096 ± 0.169
	LgP17	2.33	0.031	3.1	0.010	32.5 ± 30.0	0.051 ± 0.021	0.040 ± 0.019	0.501 ± 0.129	−0.128 ± 0.141
	LgP27	2.03	0.022	2.4	0.010	0.9 ± 3.3	0.036 ± 0.018	0.023 ± 0.013	0.368 ± 0.117	−0.178 ± 0.109
	LgP28	−10.00	0.013	2.88	0.012	4.1 ± 8.6	0.039 ± 0.018	0.026 ± 0.016	0.389 ± 0.134	0.140 ± 0.151
Taste Buds 99.40	LgP5	2.96	0.022	4.42	0.001	83.2 ± 27.1	0.166 ± 0.059	0.126 ± 0.063	32.217 ± 16.806	−4.636 ± 13.777
	LgP18	1.79	0.043	3.96	0.001	25.4 ± 2.5	0.130 ± 0.046	0.004 ± 0.006	0.608 ± 6.723	24.912 ± 5.484
	LgP25	1.74	0.045	3.4	0.002	1.3 ± 5.0	0.075 ± 0.029	0.048 ± 0.028	21.008 ± 7.013	10.179 ± 6.167

Trait Mean (μ)	Linkage Group	SIM LOD	P value	MIM LOD	P value	QTL Position (cM)	PEV	PEVad	Subst.effect (d)	Heter.effect (h)