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Demonstrating stratification in a European American population

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Population stratification occurs in case-control association studies when allele frequencies differ between cases and controls because of ancestry. Stratification may lead to false positive associations, although this issue remains controversial[1–4]. Empirical studies have found little evidence of stratification in European-derived populations, but potentially significant levels of stratification could not be ruled out[5–7]. We studied a European American panel discordant for height, a heritable trait that varies widely across Europe[8]. Genotyping 178 SNPs and applying standard analytical methods[6,9–11] yielded no evidence of stratification. But a SNP in the gene *LCT* that varies widely in frequency across Europe[12] was strongly associated with height ($P < 10^{-6}$). This apparent association was largely or completely due to stratification; rematching individuals on the basis of European ancestry greatly reduced the apparent association, and no association was observed in Polish or Scandinavian individuals. The failure of standard methods to detect this stratification indicates that new methods may be required.

We created a European American case-control panel comprised of 1,057 individuals ranked in the 5th through 10th percentiles for adult height and 1,132 individuals ranked in the 90th through 95th percentiles for adult height (**Table 1**). All individuals were born in the US and were self-described "white" or "Caucasian," and all of their grandparents were born in either the US or Europe. Height has previously been associated with ancestry in an admixed population[13]. Because height also varies across European populations[8], we thought that this panel, despite the cases and controls being matched by age, country of birth (US) and self-described ethnicity, might nevertheless be vulnerable to stratification.

To assess stratification in this panel, we genotyped two sets of SNPs in a representative sample (192 tall and 176 short subjects) from the panel. We first genotyped 111 unlinked missense and noncoding SNPs from a set of markers used previously to examine stratification[6]. We calculated the χ^2 association statistics for these 111 SNPs and compared them with the expected χ^2 distribution under the scenario of no stratification[11]. The median χ^2 value was 0.37 and the mean χ^2 value was 0.96 in the sample (**Table 2**); if stratification were present,

Table 1 Population samples

	European American[a]				Polish[a]				Scandinavian[b]			
	Short		Tall		Short		Tall		Short		Tall	
	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
n	507	550	587	545	276	236	238	268	81	85	83	85
Age[c] (y)	57 ± 9	55 ± 10	56 ± 9	54 ± 10	55 ± 10	56 ± 9	54 ± 9	55 ± 10	35 ± 7	36 ± 8	34 ± 7	34 ± 8
Height[c] (cm)	167.1 ± 1.4	153.2 ± 1.5	186.9 ± 2.0	172.0 ± 1.8	164.7 ± 1.9	153.4 ± 1.1	180.9 ± 1.3	169.6 ± 0.9	171.7 ± 3.8	160.1 ± 0.9	183.7 ± 4.0	170.4 ± 3.4

[a]The European American and Polish samples are case-control studies with subjects ranking in the 5th through 10th percentiles in adult height (short) and in the 90th through 95th percentiles in adult height (tall). [b]The Scandinavian sample consists of parent-offspring trios in which subjects below the median height in the sample are considered short and subjects above the median height are considered tall. [c]mean ± s.d.

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Table 2 No evidence for stratification using standard methods

		χ^2 values[a]		Estimates of stratification parameters[b]		
	SNPs	Median	Mean	λ_{max}	λ	*P*
Random SNPs	111	0.37	0.96	3.21	1	0.61
AIMs	67	0.58	0.95	–	–	0.61
Total	178	0.49	0.95	–	–	0.66

[a]Expected values are median $\chi^2 = 0.45$ and mean $\chi^2 = 1.00$ if no stratification is present. [b]The value λ is the inflation factor of association statistics due to stratification for a sample equivalent in size to the full European American panel, and λ_{max} is the upper 95% confidence bound on this value[6].

one would expect the median χ^2 value to be >0.45 and the mean χ^2 value to be >1. Therefore, we observed no evidence of stratification with these 111 markers.

We next carried out further tests of stratification using ancestry informative markers (AIMs) that have large allele frequency differences between Africa and Europe[14]. AIMs are useful in estimating admixture and detecting stratification in populations such as African Americans[15–17]. Because AIMs that differ in allele frequency between populations such as Europeans and Africans are more likely to differ within those groups as well[14], we reasoned that these markers may also be more powerful than random markers at detecting stratification in populations such as European Americans that have no strong recent history of admixture. We genotyped 67 AIMs in the height sample but again discerned no evidence for stratification (median $\chi^2 = 0.58$, mean $\chi^2 = 0.95$; **Table 2**). To describe the degree of stratification in the height sample that could be consistent with our data, we used the data for the 111 random SNPs to estimate the previously described[6] parameter λ. The best estimate of λ is 1, indicating that there is no inflation in association statistics due to stratification in this sample (**Table 2**).

Finally, we also used the program *structure*[18] to test for population structure in this sample. This method uses genotype data to search for evidence of subpopulations in the sample; if there are multiple subpopulations, cases and controls can be rematched on the basis of this information[18–20]. On the basis of data from the random SNPs and AIMs, the panel seemed to be one population with no outliers; this result was consistent across a range of numbers of hypothetical subpopulations (data not shown).

From these results, we conclude that standard approaches detect no evidence of population stratification in the height panel. But detection of stratification (or, equivalently, observing false positive associations due to stratification) requires not only the presence of individuals from different populations but also alleles that vary in frequency among those populations. The SNPs we typed (including the AIMs) might not vary sufficiently among European populations to allow detection of stratification. We hypothesized that a marker with a wider spread in allele frequency among European populations that differ in average height might seem to be falsely associated in the European American panel.

To test this hypothesis, we genotyped the *LCT* –13910C→T polymorphism (rs4988235), which has a wide variation in allele frequency along a cline that roughly parallels the variation in average height among European populations[12]. In particular, the frequency of the T allele of this SNP, which is associated with lactase persistence[21], is 5–10% in southern Europe and 70–80% in northern Europe[12]. We analyzed this SNP in the full European American panel. Notably, the T allele was strongly associated with tall stature (**Table 3**; $P = 3.6 \times 10^{-7}$, odds ratio (OR) = 1.37, $\chi^2 = 28.2$). The association was so strong that this SNP was out of Hardy-Weinberg equilibrium (HWE), which we confirmed was not due to genotyping error. Because the most likely estimate of λ for this sample was equal to 1, standard approaches would not require adjustment of this association for stratification. Even after a very conservative correction for possible stratification (dividing the χ^2 statistic by λ_{max}, which for this sample is 3.21), the association remained significant ($P = 0.003$; corrected $\chi^2 = 8.8$).

The association between the *LCT* marker and height could be valid or could be a false positive result due to undetected stratification. To distinguish between these possibilities, we examined the relationship between height and grandparental ancestry and the relationship between grandparental ancestry and allele frequency with respect to *LCT* –13910C→T. We observed that both height and *LCT* –13910C→T were correlated with grandparental ancestry along an approximately northwestern-to-southeastern axis in Europe (**Fig. 1**). To determine

Table 3 A strong association of *LCT* –13910C→T and height is reduced by rematching subjects on the basis of ancestry

			Origin of grandparents[a]			
		All	Four US-born	Southeastern	Northwestern	Combined[b]
N	Total	2,179	1,282	354	543	–
	Tall	1,123	645	127	351	–
	Short	1,056	637	227	192	–
LCT –13910 genotype counts[c]	Total	392:918:869	142:543:596	182:141:31	68:233:243	–
	Tall	161:474:489	66:265:314	54:55:18	41:154:157	–
	Short	231:444:380	76:278:282	128:86:13	27:79:86	–
Hardy-Weinberg *P*	Total	5.6×10^{-7}	0.57	0.89	0.89	–
	Tall	0.03	0.66	0.81	0.92	–
	Short	2.5×10^{-5}	0.86	0.96	0.45	–
Association *P*		3.6×10^{-7}	0.098	0.0016	0.71	0.0074
OR (95% c.i.)[d]		1.37 (1.22–1.54)	1.15 (0.97–1.36)	1.70 (1.22–2.38)	1.05 (0.81–1.37)	1.19 (1.05–1.36)

[a]Subjects were rematched on the basis of their grandparental countries of origin. [b]Data for the three subgroups were combined using a Mantel-Haenzsel test[22]. [c]Genotype counts are CC:CT:TT. The T allele of *LCT* –13910 is associated with lactase persistence. [d]ORs reflect the effect of the T allele on tall stature. c.i., confidence interval.

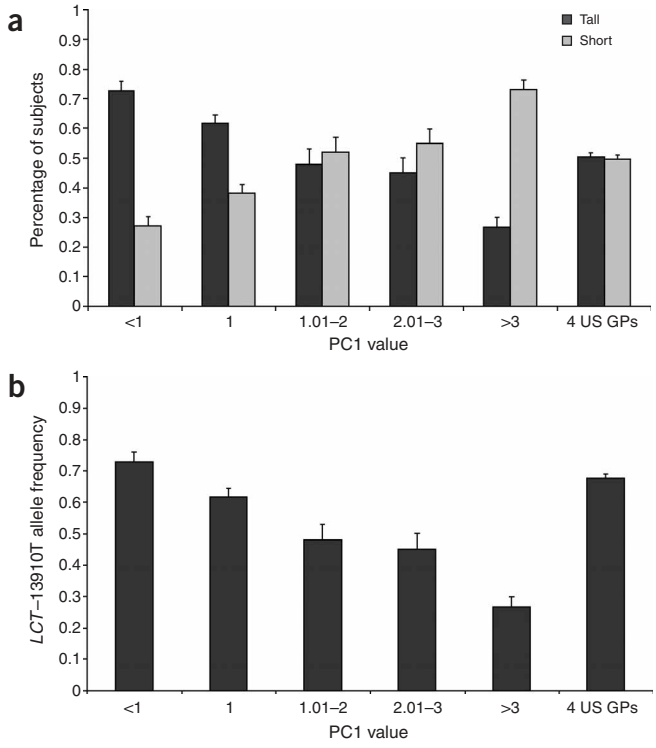

Figure 1 The relationship between European ancestry, height and *LCT* –13910C→T allele frequency in the European American panel. The percentage of tall and short subjects (**a**) and frequency of *LCT* –13910T (**b**) are plotted versus European Ancestry on the basis of PC1 (ref. 29). Low PC1 values approximate high northwestern European ancestry, and high PC1 values approximate high southeastern European ancestry. The bars indicate mean values for each of five ranges of PC1 values as well as for subjects with four US-born grandparents (4 US GPs). Error bars indicate one standard deviation.

whether these correlations partly or completely explain the apparent association, we further matched the cases and controls in the full height panel on the basis of grandparental ancestry. This rematching strategy gave us three subsamples: subjects with four US-born grandparents, subjects with predominantly southeastern European ancestry and subjects with predominantly northwestern European ancestry (**Table 3**). All three subsamples were in HWE with respect to *LCT* –13910C→T. When we combined the data from the three subgroups by a Mantel-Haenzsel test[22], the association was much less significant ($P = 0.0074$) than it was before the sample was rematched on the basis of ancestry. This marked diminution in association suggested that the initial association was largely or completely due to stratification and that the persistent association in the southeastern subgroup may be due to residual stratification not corrected with our matching scheme. Permutation testing indicated that this reduced association was due to improved matching and reduced stratification rather than to division into subgroups.

To investigate further whether the apparent association of *LCT* –13910C→T and height was due to stratification, we genotyped this SNP in two more study samples (**Table 1**). The first sample, a case-control study from Poland, was identical in design to the European American study. When we typed *LCT* –13910C→T in this sample, we observed no trend or association ($P = 0.92$, OR = 0.99; **Table 4**). These data exclude with >99% confidence an association with the OR observed in the US sample (OR = 1.37) and with 98% confidence a more conservative estimate of that association (OR = 1.22). We also genotyped this SNP in a set of Scandinavian parent-offspring trios ascertained for waist-hip ratio for whom we had height data; being family-based, this sample is immune to stratification[23]. We observed no association of *LCT* –13910C→T and height using Allison's TDTQ4 method[24] ($P = 0.43$, odds ratio=0.91; **Table 4**) or using the QTDT software package[25] ($P = 0.93$). The failure to detect even a trend toward association in the Scandinavian panel supports the results from the Polish panel. Taken together, these results strongly suggest that the association observed in the European American sample was largely or completely due to stratification.

To our knowledge, this is the first demonstration of stratification in a real, apparently well matched sample comprised entirely of individuals with recent European ancestry. Furthermore, standard methods did not detect evidence of stratification in this sample. The markers used in these methods probably do not vary widely within Europe and are less suitable for predicting the behavior of markers such as *LCT* –13910C→T. It is not known how many markers have widely varying allele frequencies among closely related populations, or how many phenotypes vary across Europe. Nevertheless, this clear example of stratification raises the strong concern that markers with less marked frequency variation than *LCT* –13910C→T and phenotypes less strongly stratifying than height could also give rise to false positive associations due to stratification, and that this stratification would be hard to detect using standard approaches. The recent demonstration of population substructure in Iceland, which was previously thought to be 'homogeneous', underscores the need for sensitive measures to detect stratification[26].

We suggest two new, complementary approaches for assessing stratification. First, we advocate the identification of markers that, like *LCT* –13910C→T, vary in frequency among closely related populations. Such markers could be informative in detecting subtle but important levels of population stratification in apparently homogeneous study samples and could potentially be used for rematching

Table 4 No association of *LCT* –13910C/T and height in other European populations

		Polish	Scandinavian	Combined
Genotypes (CC:CT:TT)	Tall	166:251:86	–	–
	Short	174:235:96	–	–
Transmissions of T allele (T:U)[a]	Tall	–	65:68	–
	Short	–	76:66	–
P		0.92	0.43	0.58
OR (95% c.i.)[b]		0.99 (0.83–1.18)	0.91 (0.72–1.15)	0.96 (0.83–1.11)

[a]For the TDT data in the Scandinavian trios, T is the count of transmissions of the –13910T allele from heterozygous parents and U is the count of transmissions of the C allele. [b]ORs reflect the effect of the T allele on tall stature. c.i., confidence interval.

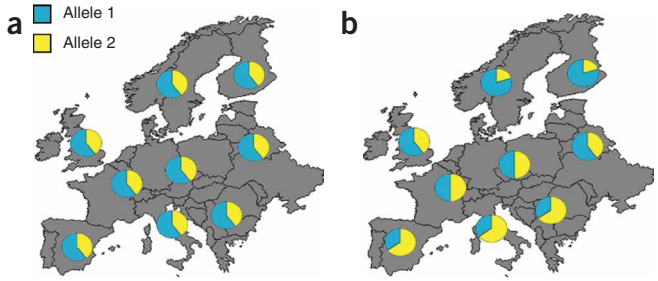

Figure 2 A complementary approach for assessing the likelihood of an association being explained by stratification. If the allele frequencies of an associated marker are similar throughout a range of closely related possible source populations, in this case, Europe (**a**), then the association is less likely to be a false positive due to stratification. If allele frequencies differ widely among closely related populations that could be represented in the study sample (**b**), then the possibility of a false positive association due to stratification must be strongly considered.

samples if detailed ancestry is unavailable. Finding these particularly informative markers will require surveying the frequency of large numbers of variants in many closely related populations. Current 'ancestry informative' markers will probably not be sufficiently informative in populations that are not strongly admixed.

Second, just as some markers are more useful in detecting stratification, certain markers are more likely to be falsely associated due to stratification. The effects on stratification of marker allele frequency[11] and a nonsignificant trend in the effects of the class of marker (missense or random)[6] were previously described. We propose a different assessment of the susceptibility to stratification based on the variation in allele frequencies in closely related populations. For example, if the frequency of an associated marker is constant across Europe, then the association is unlikely to be due to stratification in a European American population. Conversely, if the marker frequency varies widely across Europe, the result is more likely to be due to stratification (**Fig. 2**). Thus, we recommend that the frequencies of associated markers should be measured in different subpopulations that might be represented in the study. Furthermore, we found that having grandparental-country-of-origin information can be valuable for assessing stratification. If a marker does vary in frequency across relevant populations, then the grandparent-country-of-origin data could be used to rematch the sample into subgroups along a cline similar to that of the associated marker frequencies, as we did with *LCT* –13910C→T. If the association is a true positive, it should persist even when each subgroup is analyzed separately and then recombined using a Mantel-Haenzsel test. Using this approach to minimize false positive associations from stratification will require large samples from many distinct but closely related populations in which allele frequencies of associated markers can be measured accurately and new statistical methods to use this allele frequency data to detect and correct for population stratification appropriately.

METHODS

DNA samples. We selected the samples in the European American and Polish height panels (**Table 1**) from samples collected by Genomics Collaborative. These two studies are case-control studies in which the tall subjects were in the 90th through 95th percentiles in adult height and the short subjects were in the 5th through 10th percentiles in adult height. Grandparental and parental countries of origin are known for these samples. The short and tall subjects in the European American panel were also matched according to region of residence in the US. When we analyzed *LCT* –13910C→T in each region of the US separately and combined the data using a Mantel-Haenzsel test, the results were similar to those observed when we analyzed the panel as a whole ($P = 5.5 \times 10^{-6}$, OR = 1.33; ref. 22).

Parent-offspring trios (**Table 1**) from Finland and Sweden were ascertained as having a waist-hip ratio in the upper quintile or lower decile, as described previously[27]. Height data for these samples are known; the median heights for male and female offspring were determined and used to divide the offspring into tall (above the median) and short (below the median) groups. All subjects gave informed consent, and the project was approved by the institutional review board of the Children's Hospital, Boston.

Genotyping and SNPs. All genotyping was done using the mass spectrometry–based MassArray platform (Sequenom) as described elsewhere[28]. We designed primers using SpectroDesigner (Sequenom). For efficiency, we genotyped 128 SNPs in a random subset of the European American height panel initially to test for stratification; 80 SNPs were missense SNPs and 48 SNPs were noncoding SNPs[6]. Of these SNPs, 111 passed quality control. We genotyped 90 AIMs[6,15] in the European American panel, 67 of which passed quality control. To pass quality control, a SNP must have a genotype success rate >75%, a minor allele frequency >1% and one or fewer discrepancies among duplicate samples. Excluding SNPs (one random SNP and four AIMs) with Hardy-Weinberg *P* values <0.01 and high genotyping percentages did not substantially change the results. To confirm that the deviation from HWE for *LCT* –13910C→T was not due to genotyping error, we also genotyped a second SNP (rs182549), which was in nearly perfect linkage disequilibrium with *LCT* –13910C→T in our sample, was also associated with height and was similarly out of HWE in the original population (data not shown).

Data analysis. For the random SNPs and AIMs genotyped in the European American height panel, we computed χ^2 values for association for the working SNPs and calculated *P* values for the presence of stratification as previously described[6,11]. We calculated the stratification parameters λ and λ_{max} for the full European American sample as previously described[6,9]. λ is the factor by which association statistics are typically inflated in the presence of stratification, and λ_{max} is the upper 95th percentile confidence bound on λ. We also calculated the most likely value of λ assuming that *LCT* –13910 and the random SNPs were chosen from the same distribution. This value was 2.5, which is less than λ_{max}. The likelihood of observing our data under this model was 300 times less likely than observing our data under the model where *LCT* –13910 was drawn from a different distribution than the random SNPs. Therefore, the association with *LCT* –13910 is not consistent with the degree of stratification estimated by the random SNPs. We used an even more conservative correction, λ_{max}, to illustrate this point. We ran the program *structure*[18] using the suggested parameters and assuming one to six population subgroups.

Rematching of European American samples. We used the grandparental-country-of-origin information on the European American subjects to separate out all subjects with four US-born grandparents because we did not have ancestry information to subdivide this group further. We then subdivided the remaining subjects as follows. First, we assigned each non-US-born grandparent a value based on his or her country of origin that was derived from Cavalli-Sforza's principal component analysis of Europe using principal component one (PC1), because this represents a geographic cline that roughly approximates the cline in *LCT*–13910C→T frequencies[29]. Next, we averaged the values for a subject's grandparents to assign each subject a PC1 value. We divided the subjects into two groups roughly corresponding to southeastern and northwestern Europe (above and below the mean PC1 value, respectively).

Permutation testing of rematching scheme. We randomly permuted individuals 1,000 times between the three subgroups and carried out the combined association analysis for each permutation. In the 1,000 permutations, the pooled Mantel-Haenzsel *P* values remained between 2.4×10^{-7} and 8.9×10^{-7}, similar to the *P* value observed before rematching (3.6×10^{-7}), indicating that the loss of significance we observed with our matching scheme based on ancestry was not due to creating subgroups and recombining the data.

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COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the *Nature Genetics* website for details).

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