

## ORIGINAL INVESTIGATION

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## Geographic homogeneity and non-equilibrium patterns of mtDNA sequences in Tuscany, Italy

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**Abstract** The geographical distribution of 49 mtDNA sequences from 22 localities in Southern Tuscany, Italy, was studied by molecular analysis of variance, by a new spatial autocorrelation statistic specifically designed for sequence data and by reconstructing genealogies of haplotypes. All these methods indicated a high homogeneity of populations. Nevertheless, genetic variability showed significant departure from equilibrium expectations, in agreement with the predicted effects of a population expansion. We suggest that a past population expansion that was probably associated with a migrational wave and with local gene flow between localities prevented spatial structuring in Southern Tuscany.

### Introduction

The frequencies of different neutral variants of the same protein randomly fluctuate by drift in finite populations. In subdivided populations, this process can be considered to be almost independent in different demes. As a consequence, differences in allele frequencies randomly accumulate following subdivision, at a rate inversely related to the levels of gene flow and to the effective population size

(Slatkin 1987). Population genetic theory of subdivided populations has been developed with this type of process in mind and therefore several methods are available for identifying, describing and interpreting the spatial variability of allele frequencies (see, for example, Wijsman and Cavalli-Sforza 1984).

Molecular biological techniques have recently shown that highly variable DNA regions are present in humans and other organisms. These regions are usually non-coding DNA sequences and, as predicted by theory (Ewens 1972), the number of different alleles in a sample is often high. In interpreting data of this kind, the mutational process can no longer be neglected, since it concurs with random drift in the establishment of genetic differences among subpopulations. The presence of different private haplotypes in different subpopulations can be regarded as a clear example of population diversity essentially reflecting the consequences of mutation.

When highly polymorphic loci are studied, therefore, both the frequencies of the different haplotypes and their genealogical relationships ought to be considered. Few statistical methods are available for studying population structure at this level. In this paper, three such techniques have been used to analyse the geographical structuring of mtDNA sequences in southern Tuscany, Italy.

Southern Tuscany represented the centre of the Etruscan civilization, whose origin was probably related to the Palaeolithic peopling of Europe (Francalacci et al. 1996). The Etruscan language does not belong to the Indo-European family and so the impact, on this region, of Neolithic migrational waves (Cavalli-Sforza et al. 1993), probably involving people speaking Indo-European languages (Renfrew 1987; Barbujani et al. 1994, 1995b) remains unclear. It has been suggested, however, that the populations of Tuscany have remained in relative isolation from the surrounding Italians (Renfrew 1993), at least starting from the seventh century BC (the oldest historical dating of the Etruscan culture). This hypothesis is supported by linguistic evidence (Devoto 1977) and, in part, by the analysis of gene frequencies (Piazza et al. 1988; Barbujani and Sokal 1991). The population dwel-

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ling in this region may have been stable for a long period of time and the Etruscan civilization may have developed in demographic and cultural continuity with the earliest, so-called Villanovan, culture (Pallottino 1984). In the absence of more detailed palaeodemographic information, DNA sequence analysis could provide insights into the genetic structure of this population and hence into its evolutionary history.

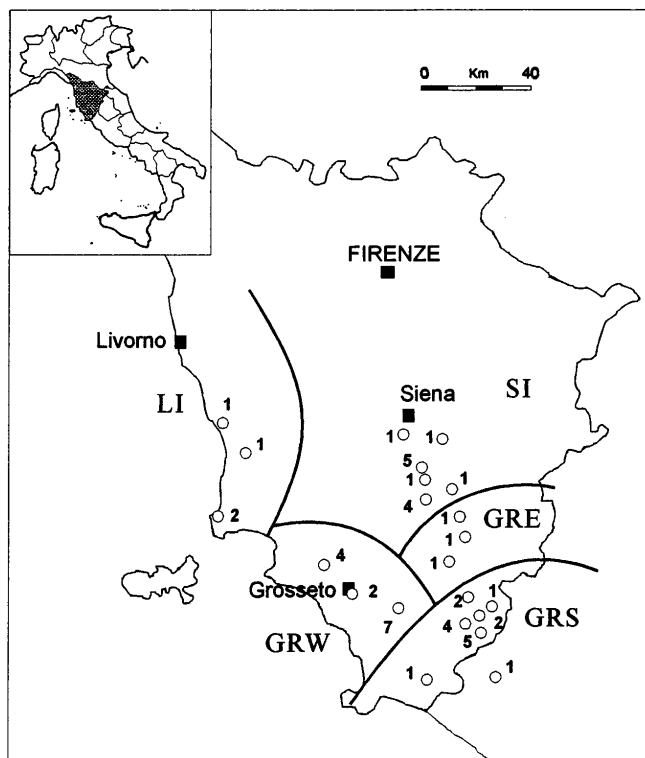
We have tried to address four basic evolutionary questions. (1) Are the various localities in this study genetically different? (2) Is there any pattern in the distribution of haplotypes in the geographical region? (3) Is there any evidence of migrational processes determining the observed distribution of haplotypes? (4) Do the populations appear to be near equilibrium or far from it?

## Materials and methods

### The data

The mtDNA control region (segment 1 plus segment 2: 780 bp) was sequenced in 49 individual mtDNAs coming from 22 localities of Southern Tuscany (Fig. 1). Details of the data set and of the molecular techniques employed for typing are reported elsewhere (Francalacci et al. 1996).

The limited sample size has made it impossible to analyse the relationships between individual localities in some cases. Therefore, each locality has been assigned to one of five geographical regions, coded as follows (see Fig. 1): Livorno (Leghorn; LI),



**Fig. 1** Geographic location of the samples. Numbers correspond to the sample size. Five regions are coded as follows: Livorno (Leghorn; LI), Siena (SI), Grosseto East (GRE), Grosseto South (GRS) and Grosseto West (GRW)

Siena (SI), Grosseto East (GRE), Grosseto South (GRS) and Grosseto West (GRW). The towns of Livorno, Siena and Grosseto are the main centres of the area. Such an administrative subdivision reflects historical differences dating back to the Middle Ages. More remote subdivision is difficult to establish with reasonable accuracy. We have chosen to regard the GRE and GRW localities as belonging to distinct regions, because they are situated in flat and hilly areas, respectively, and both are separated from the GRS localities by the Monte Amiata.

A previously published specific statistical method was applied to each of the four questions listed in the Introduction. Thus, data were subjected to four different kinds of analysis, namely: (1) analysis of molecular variance (AMOVA); (2) spatial autocorrelation analysis; (3) reconstruction of gene genealogies and estimation of the minimum numbers of migrational events compatible with them; (4) tests of mutation-drift equilibrium.

### Analysis of molecular variance

Genetic variation was first subdivided into three hierarchical levels by means of AMOVA (Excoffier et al. 1992). By this method, the relative amount of sequence diversity was quantified among individuals within localities, among localities within regions, and among the five regions defined above. In addition,  $\Phi$  statistics were computed. These indices can be considered as molecular equivalents of Wright's  $F$  statistics (Wright 1951), in that they summarize genetic diversity at various (in our case, three) levels of hierarchical subdivision.

The statistical significance of each variance component and of each  $\Phi$  value was evaluated by a permutational analysis. The 49 individuals were randomly reassigned to the localities in the study, keeping local sample sizes constant. After such a reallocation, the respective genetic variances and  $\Phi$  statistics were recalculated. The entire procedure was repeated as many times as necessary (1000 times here). In this way, null distributions of the statistics of interest could be constructed under the hypothesis of random spatial distribution of haplotypes. The observed variances were then compared with such empirical null distributions, thus assessing their significance.

### Spatial autocorrelation analysis

Spatial autocorrelation analysis was performed by calculating the so-called AIDA statistics. AIDAs are autocorrelation coefficients for DNA analysis (Bertorelle and Barbujani 1995) and may be useful for summarizing complex patterns in space. The statistic that we chose ( $I$ ) was calculated by comparing haplotypes pairwise, in arbitrarily chosen distance classes. The level of genetic resemblance within each class was measured on a scale between  $-1$  and  $+1$ , i.e. in a similar manner to a correlation coefficient. When, in a given distance class, the pairs of haplotypes compared tended to resemble each other,  $I$  was positive; when they tended to differ,  $I$  was negative. The expected value was close to 0 under the null hypothesis of no geographical structuring, i.e. when the levels of genetic resemblance in the class being considered were the same as those between random pairs of haplotypes, regardless of their distance.

In this study, DNA sequences were compared at seven spatial lags. The first one (distance 0) corresponded to the 63 comparisons between haplotypes sampled in the same locality and represented a measure of intra-population genetic relatedness. All other classes included comparisons at increasing distances. The class limits were chosen in such a way that each distance class contained approximately the same number of comparisons. Sequence similarity was summarized in a "correlogram" as a function of the geographic distance between the haplotypes being compared. Significance of autocorrelation indices was evaluated by a randomization procedure, similar to that described for AMOVA. The 49 haplotypes were randomly re-allocated 1000 times; for each time, *pseudo-I* values were calculated whose distribution was used to

evaluate the confidence limits around the value expected under the hypothesis of no spatial pattern.

#### Numbers of migrational events

Relative levels of gene flow between each pair of regions were estimated by a parsimony method (Slatkin and Maddison 1989), based on the reconstruction of genealogies of haplotypes. Each genealogy included only the haplotypes sampled in two different regions. Since five geographical regions were previously defined, 10 ( $5 \times 4/2$ ) genealogies were reconstructed, using the neighbour-joining tree method (Saitou and Nei 1987). Genetic distances between haplotypes were estimated on the basis of the pairwise sequence differences, assuming a Kimura two-parameter model (Kimura 1980) with transversions weighted ten times as much as transitions. From each of the ten trees, a minimum number of migration events ( $s$ ) consistent with the genealogy and with the geographic origin of the individuals in it was estimated. The value of  $s$  depends on the clustering in the tree of haplotypes with similar geographic origin: the higher the clustering, the lower the minimum number of migration events necessary to account for the topology of the tree. If the haplotypes sampled in the two regions form clearly separated clusters in the tree, one can infer that little migration has occurred between the regions. Indeed, it has been established that  $s$  is directly related to the level of gene flow (Slatkin and Maddison 1989). The value of  $s$  is, however, constrained by the sample size. The maximum number of migrational events that can be estimated from a genealogy,  $s_{max}$ , simply corresponds to the size of the smaller of the two samples under consideration. To standardize comparisons, therefore, relative  $s_r$  values have to be computed by dividing  $s$  by the respective  $s_{max}$ . Each value of  $s_r$  may then be viewed as a measure of gene flow between two regions, relative to the maximum amount that this method is able to detect.

#### Testing for equilibrium

In the final step of the analysis, all the 49 sequences were pooled. Departure from neutral expectations was assessed by the tests of Tajima (1989a) and Ewens-Watterson (Ewens 1972; Watterson 1978), each of which depends on different null models.

Assuming an infinite allele model, the Ewens-Watterson test checks whether the observed homozygosity  $F$  (equal to the sum of the squared haplotype frequencies) falls in the  $(1-\alpha)\%$  of the distribution expected under neutrality, where  $\alpha$  is the significance level chosen. This distribution is fully specified by the sample size and the number of observed alleles and can be determined by simulation following the algorithm in Fuerst et al. (1977).

The neutrality test of Tajima (1989a) assumes an infinite-site model. It is based on a comparison between two estimates of  $M = 2Nu$ , where  $N$  is the population size and  $u$  the mutation rate. These estimates are calculated independently from the average pairwise difference between sequences and from the number of polymorphic sites. Under neutrality, these estimates should be equal and their standardized difference  $D$  is approximately beta-distributed around 0. The observed  $D$  value can then be compared with the confidence intervals, as calculated by Tajima (1989a). Since segment 1 and segment 2 of the control region differ in their substitution rate (Vigilant et al. 1991; Wakeley 1993; Francalacci et al. 1996), neutrality tests were applied separately to the two segments.

## Results

The molecular analysis of variance (Table 1) shows that the observed variation can be almost entirely ascribed to differences within localities (94.3%). Even so, this component of the variance falls in the range expected under the null hypothesis of random allocation of haplotypes

**Table 1** Analysis of molecular variance (AMOVA).  $P$  is the probability of having a more extreme variance component and  $\Phi$  statistic, under the spatially random distribution of haplotypes. None of these values is significant at the  $P = 0.05$  level

	Variance	% total	$\Phi$ statistics	$P$
Among regions	-0.017	-0.41	$\Phi_{ct} = 0.004$	0.231
Among localities/ within regions	0.259	6.10	$\Phi_{sc} = 0.061$	0.255
Within localities	4.000	94.31	$\Phi_{st} = 0.057$	0.160

**Table 2** Spatial autocorrelation analysis.  $I$  is the spatial autocorrelation coefficient. None of the observed values is significantly different from the value expected under a randomization hypothesis.  $N$  is the number of pairwise comparisons in each distance class

Class limits (km)		$I$	$N$
Upper	Lower		
0	0.1	-0.026 (ns)	63
0.1	22.1	0.000 (ns)	187
22.1	34.7	-0.037 (ns)	171
34.7	47.6	-0.021 (ns)	178
47.6	56.0	-0.013 (ns)	160
56.0	71.3	-0.032 (ns)	177
71.3		-0.022 (ns)	210

( $P = 0.160$ ). This is a clear indication of homogeneity between populations and is confirmed by the estimates of  $\Phi$ , which are very low at all hierarchical levels considered. The negative estimate of variance observed between geographic regions (and the corresponding negative value of  $\Phi_{ct}$ ) is common in populations that are not subdivided. Slightly negative estimates such as these may occur because they are approximated by subtraction of other components of variance from the overall variance observed (Excoffier et al. 1992). In practice, this means that variance is 0 between regions, indicating that random haplotypes drawn from different regions do not resemble each other any the less than haplotypes from the same region. Of course, this value is insignificant and it should be interpreted only as another sign of genetic homogeneity of the five regions that we have defined. Thus, no significant hierarchical structuring of subpopulations is revealed by AMOVA.

The spatial autocorrelation coefficients calculated in the seven distance classes are all very close to zero. At no distance, including distance zero, do the observed values of  $I$  significantly differ from the values expected when haplotypes are randomly assigned to the sampled localities (Table 2). In other words, the probability of observing two identical or similar haplotypes is the same for all comparisons, regardless of the spatial distance between the localities from which the haplotypes come.

The absence of a spatial pattern in genetic variation is also confirmed when the five regions are pairwise compared by reconstructing the genealogies of their sequences. An example is given in Fig. 2. In most genealogies, haplotypes coming from the two regions largely in-



**Fig. 2** Neighbour-joining tree of the haplotypes in the Siena (SI) and Grosseto West (GRW) regions. Migration events inferred under parsimony assumptions are marked by arrows

**Table 3** Minimum number of migrational events, relative to its maximum value, for each pair of regions

	LI	SI	GRE	GRS	GRW
LI	–	1.00	0.67	0.75	1.00
SI		–	1.00	0.54	0.69
GRE			–	1.00	0.67
GRS				–	0.61
GRW					–
Average	0.85	0.81	0.83	0.72	0.74

termingle, with no clear clustering of individuals with similar geographical origin. This situation is reflected in the values of  $s_n$ , the relative minimum number of migrational events (Table 3), which are always larger than 0.5 and reach the maximum possible value in 4 out of 10 cases. The average values show a lower level of gene flow for GRW and GRS; this presumably reflects their marginal position in the map, the less-than-optimal environmental conditions of the former (where malaria was eradicated only recently) and the isolation of the latter.

The results of the Ewens-Watterson and Tajima tests are presented in Table 4. They were separately applied to the two segments of the control region. For both segments, the Ewens-Watterson test indicated that the observed homozygosity,  $F$ , was significantly higher than expected under neutrality. For mtDNA, homozygosity simply means genetic homogeneity. High values of  $F$  are expected when one haplotype is common, intermediate-frequency haplotypes are few or absent and there are many rare alleles (Clark 1987). Similarly, Tajima's  $D$  statistic was lower than expected for both segments, once again

**Table 4** Results of the Tajima and Ewens-Watterson neutrality tests on segment 1 and segment 2 of the control region.  $F$  is the observed homozygosity (equal to the sum of the squared haplotype frequencies). Stars represent the significance of the departures from the neutrality expectation. \*  $P < 0.05$ , \*\*  $P < 0.01$

	Tajima's $D$ statistic	$F$
Segment 1	–2.06*	0.051**
Segment 2	–1.57	0.087**

suggesting a deficit of haplotypes at intermediate frequencies. Strictly speaking, statistical significance was reached only for the longer segment of the control region. If several mutations independently occur at the same DNA site, the values of  $D$  tend to increase (Bertorelle and Slatkin 1995). Therefore, recurrent mutation cannot account for the negative  $D$  values that we report. Both Tajima's test and the Ewens-Watterson test point to a departure of the population studied from the levels of genetic variation expected when the input of new alleles through mutation is balanced by their loss through drift. The number of rare alleles is higher than expected under equilibrium.

## Discussion

Four different approaches have been used in this study to investigate the spatial distribution of human mtDNA sequences in southern Tuscany. We shall discuss the evidence that they provide in relation to the four questions listed in the Introduction.

AMOVA shows that genetic differences are small at all levels tested, namely within and between localities (corresponding to villages or towns) and between regions (corresponding to areas that can be considered as geographically homogeneous). This result does not depend on the assignment of localities to regions, as a different grouping (results not reported) does not yield higher variances. The overall sequence variation is not negligible. On the contrary, this South Tuscany sample shows higher sequence diversity than other European groups, such as Basques, Sardinians and people from the British Isles (Francalacci et al. 1996). However (question 1), the localities and the regions of this study do not appear to be genetically different.

Similarly, spatial autocorrelation analysis does not provide evidence for a correlation between the similarity of mtDNA sequence and geographic distance. No patterns seem to be present in the geographical distribution of haplotypes (question 2). In addition, the reconstruction of genealogies does not suggest that any pairs of regions have experienced the exchange of higher numbers of migrants than any others. The two southern-most regions seem to have been more isolated, which is not surprising, given the nature of their territory (question 3).

All these findings demonstrate that the Southern Tuscany population is not internally structured at the mtDNA sequence level. The individuals in the sample can there-

fore be regarded as coming from a single panmictic unit. Local populations are expected to diverge genetically if gene flow is limited for a certain period of time. As far as highly variable DNA sequences are concerned, that period must be long enough for different mutations to occur in different zones and for drift to alter allele frequencies. Thus, the population in this study shows no structure, because barriers to gene flow either were weak or have been effective for too short a time or both.

These two possibilities are not easy to disentangle. Consistent amounts of gene flow probably occurred between Southern Tuscany localities, since they are not separated by obvious barriers to human displacement. However, we have found a departure from mutation-drift expectations (question 4); this suggests that internal gene flow, generally pushing subdivided populations towards equilibrium, is not a sufficient explanation. Alternatively, or in addition, other demographic pressures seem to have marked the history of these populations. In particular, the results that we have obtained by using Tajima's test and the Ewens-Watterson test can be expected if the population experienced rapid growth in the past. Indeed, if the individuals in a population have been markedly fewer in number than at present for a long period, the relationships between different measures of genetic variability do not correspond to equilibrium expectations (Tajima 1989b; Watterson 1986). Therefore, a demographic expansion, together with local gene flow, might have been important in reducing the genetic diversity in this area.

Historical evidence points to an increase of inhabited centres in Italy following the last glacial maximum, around 18 000 years ago (Mussi 1990). In agreement with this, mtDNA restriction fragment length polymorphism (RFLP) variation in Italy is consistent with the effects of a demographic expansion in the late Palaeolithic or in the early Neolithic, i.e. between 8000 and 20 000 years before present (Barbujani et al. 1995a). The analysis of the present data set based on different statistical methods and its comparison with other European data agree with this, confirming that the population ancestral to the current Southern Tuscans may have increased substantially in or before early Neolithic times (Francalacci et al. 1996). Successive demographic events (discussed in Francalacci et al. 1996) do not seem to have had the potential for major shifts in the genetic make-up of the population. In particular, there is little evidence that Roman colonization entailed massive immigration.

Various data therefore seem to corroborate the hypothesis that demographic growth played a role in preventing divergence of mtDNA lineages in Tuscany. However, a different interpretation can be drawn from our results. Indeed, it has been shown that significant results of the Tajima and Ewens-Watterson tests might also be attributable to a response to selection (Excoffier 1990). The reason that a major role of selection seems unlikely is that the mtDNA control region is not a coding region and therefore adaptive mechanisms are not expected to affect its sequence diversity. Because mtDNA does not recombine, high levels of linkage disequilibrium exist (Stoneking

1991) and so one cannot rule out the possibility that selection at other coding regions has had some impact on diversity in the control region. Nevertheless, the high mutation rate observed in the control region (Ward et al. 1991), possibly ten times greater than in the rest of the molecule, makes this scenario unlikely (Wakeley 1993). A past population expansion remains, therefore, the simplest explanation for the findings of this study, in agreement with proposals to account for similar results in other studies (Whittam et al. 1986; Merrywether et al. 1991; Harpending et al. 1993).

Returning to the question of the peopling of Tuscany, this study cannot prove whether its ancient inhabitants were or were not genetically distinct from their neighbours. However, it does appear that important aspects of the current population structure of Tuscany have been determined during the course of a demographic increase. As we mentioned above, analysis of mitochondrial RFLPs suggests that populations expanded rapidly over much of Italy, with the probable exception of Sardinia, some time between 8000 and 20 000 years ago (Barbujani et al. 1995a). We see no reason to believe that this demographic phenomenon should have been any different for Tuscany. This leaves two possibilities open. Around 8000 years ago, or slightly less, the population of Tuscany may have expanded during the course of a large-scale continent-wide process, associated with the Neolithic spread of farming from the Levant (Ammerman and Cavalli-Sforza 1984) and a possible decrease in infant mortality (Pennington 1996). Conversely, if we place the Tuscan population's expansion in the Palaeolithic, a local demographic phenomenon seems more likely, its cause possibly lying in some sort of climate improvement (Mussi 1990).

In both cases, the analysis of mtDNA lineages gives us insights only into old historical episodes that are so ancient that their consequences are not restricted to a single area but are shared by various Italian, and perhaps European, populations. Because the first signs of the Etruscan culture are judged to be less than 3000 years old, it seems unlikely that the analysis of contemporary mtDNA will further clarify the biological characteristics of the people who developed it. On an evolutionary scale, the isolation of Southern Tuscans, previously suggested by historical, linguistic and gene frequency evidence, should therefore be regarded as a recent phenomenon that has left a clear signature only at the level of fast-evolving genetic markers, such as allele frequencies, and possibly small tandem repeat polymorphisms. The Etruscan's mitochondrial gene pool may not differ significantly from the gene pools of other ancient European populations.

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