The mystery of Etruscan origins: novel clues from *Bos taurus* mitochondrial DNA

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The Etruscan culture developed in Central Italy (Etruria) in the first millennium BC and for centuries dominated part of the Italian Peninsula, including Rome. The history of the Etruscans is at the roots of Mediterranean culture and civilization, but their origin is still debated: local or Eastern provenance? To shed light on this mystery, bovine and human mitochondrial DNAs (mtDNAs) have been investigated, based on the well-recognized strict legacy which links human and livestock populations.

In the region corresponding to ancient Etruria (Tuscany, Central Italy), several *Bos taurus* breeds have been reared since historical times. These breeds have a strikingly high level of mtDNA variation, which is found neither in the rest of Italy nor in Europe. The Tuscan bovines are genetically closer to Near Eastern than to European gene pools and this Eastern genetic signature is paralleled in modern human populations from Tuscany, which are genetically close to Anatolian and Middle Eastern ones.

The evidence collected corroborates the hypothesis of a common past migration: both humans and cattle reached Etruria from the Eastern Mediterranean area by sea. Hence, the Eastern origin of Etruscans, first claimed by the classic historians Herodotus and Thucydides, receives strong independent support. As the Latin philosopher Seneca wrote: *Asia Etruscos sibi vindicat* (Asia claims the Etruscans back).

**Keywords:** *Bos taurus*; *Homo sapiens*; mitochondrial DNA; Etruscans; population genetics

1. INTRODUCTION

Historical documents and archaeological remains indicate that, compared with contemporaneous Italic *gentes*, Etruscans were more developed in terms of political and social structure, complexity of religion, ability in the arts, technologies (metallurgy in particular) and sailing, as demonstrated by their powerful military and trading fleet (Cristofani 1999).

Etruscan ethnogenesis still remains to be unravelled. Archaeologists either support local development with Eastern cultural influences (Barker & Rasmussen 1998) or an Eastern Mediterranean provenance (Beekes 2003). The second hypothesis is upheld by more than 30 classical authors, particularly Herodotus (1.94) and Thucydides (IV, 109) in their fifth century BC writings. Philological evidence is also controversial. Many support the non-Indo-European root of the Etruscan language (Pallottino 1989; Briqk 2000), while novel interpretations indicate a Semitic origin (Semerano 2003). So far, analyses on human mitochondrial DNA (mtDNA) are not conclusive. In fact, according to different authors, present Tuscans are considered as either modern descendants of Etruscans (Franchalacci *et al.* 1996) or having a weak genetic relationship with them (Belle *et al.* 2006). Moreover, mtDNA obtained from ancient Etruscan bones (Vernesi *et al.* 2004) points out to a possible genetic flow from Eastern Mediterranean regions. This is the geographical area where, ca 10 000 years ago, animal and plant domestication and the onset of agriculture determined a quantum leap in the evolution of human culture. Since then, during the diffusion of agriculture out of the Fertile Crescent, domestic animals together with crop seeds silently witnessed the dispersal of farmers (Bruford *et al.* 2003; Bellwood 2005), whose genetic impact on modern Europeans is, however, rather controversial (Haak *et al.* 2005).

After domestication, survival and diffusion of *Bos taurus* completely depend on humans; thus, the phylogeographic patterns of cattle genetic diversity should mirror human activities or movements and may provide information complementary to archaeological and anthropological data (Kidd & Cavalli-Sforza 1974).
2. MATERIAL AND METHODS

(a) *Bos taurus* mtDNA sequences

Genealogical information recorded in breed herd books was exploited to sample 164 Italian and 12 Greek *B. taurus* having minimal maternal relationships. A 271 bp fragment of the bovine mtDNA control region, corresponding to positions 16,042–16,313 of *B. taurus* mitochondrial genome reference sequence (accession number V00654), was amplified and sequenced in both forward and reverse directions according to Troy et al. (2001). Moreover, a total of 467 mtDNA sequences from European, Near Eastern and African *B. taurus* (Cymbron et al. 1999; Troy et al. 2001; Miretti et al. 2004; Beja-Pereira et al. 2006) were downloaded from GenBank and included, with the 176 new cattle sequences, in a bovine mtDNA dataset comprising 643 sequences.

(b) *Homo sapiens* mtDNA sequences

A dataset comprising 9590 human mtDNA HVSI sequences (Achilli et al. in press), ranging from position 16,024–16,383 of the Cambridge reference sequence, was used (table S2 of electronic supplementary material for more details). Haplotype frequencies were calculated considering 16 major haplogroups: H; R0; J; K; N1; N2; HV0; T; U1; U2e; U3; U4; U5; U*; X; and ‘other’.

Novel *B. taurus* sequences were submitted to GenBank (accession numbers AY700402–AY700565 and DQ518325–DQ518336).

Details on number of individuals, breed of origin and grouping according to geographical criteria are shown in table S1 of electronic supplementary material.
The geographical areas are considered: Egypt, the Middle East, Anatolia, the Balkan Peninsula, Eastern Europe (Balkan Range and Eastern Plains), Mainland Europe, Western fringe of Europe, Britain, Iberian Peninsula, Northern Italy, Tuscany and Southern Italy. Haplotypes are shown as coloured circles and unsampled intermediates as small dots. Circle diameter is proportional to haplotype frequency. Haplogroups are named according to the current nomenclature (Troy et al. 2001; Mannen et al. 2004) and are defined by substitutions at positions (+16,000) indicated in the skeleton network.

(c) Statistical analyses

Mean numbers of pairwise nucleotide differences were calculated on bovine sequences using ARLEQUIN v. 2.000 (Schneider et al. 2000). Haplotype variation was depicted by median joining networks (NETWORK v. 4.1 program; http://www.fluxus-engineering.com), resolving the reticulations through a maximum-parsimony criterion. Reynolds genetic distances (Reynolds et al. 1983) were computed by ARLEQUIN v. 2.000 and represented in two dimensions by multi-dimensional scaling (MDS; Kruskal 1964), using the software STATISTICA v. 7.0.

Admixture coefficients were calculated with ADMIX v. 2.0, which computes the relative contribution of any number of parental populations to a derived hybrid population using the estimator $mY$ (Dupanloup & Bertorelle 2001). The $mY$ estimator incorporates both the allelic frequencies and the effect of mutation since an instantaneous admixture event. No drift is assumed after admixture. In particular, ADMIX v. 2.0 has been used to obtain figures allowing us to quantitatively compare the composition of the different gene pools, rather than to make inferences about the admixture event itself.

Unexpectedly, six Italian breeds fell outside the Western European group, and seemed to have a genetic tie with breeds from the Balkans, Anatolia and Middle East. Notably, four of the aforementioned six breeds (Chianina, Calvana, Maremmana and Cabannina) originated in Tuscany and Eastern Liguria. These two Italian regions closely overlap ancient Etruria, where the Etruscans were settled.

A certain degree of affinity with Turkish and southern Anatolian breeds was also shown by Cinisara and Rendena. The former is autochthonous in Sicily, an important crossroad for Eastern Mediterranean cultures in the first millennium BC, as the remains of Punic and Greek colonies testify. The latter is raised in the mountain region along the Adige River (northeast Italy), where in ancient times the Raeti settled. Intriguingly, archaeological remains suggest that Raeti shared a common origin with Etruscans, because they had been speaking a proto-Etruscan language at least since the beginning of the Iron Age onwards (van der Meer 2004).

The position of the Retinta, far from other Iberian B. taurus (Barrenda, Alistana) and close to the Egyptian breeds, confirmed the uneven introgression of African mtDNA into Iberian breeds (Cymbron et al. 1999; Miretti et al. 2004; Beja-Pereira et al. 2006).

Further analyses were conducted grouping B. taurus populations according to the geographical area of origin (figure 1b). Within Italy, three main regions were defined, namely Tuscany, Northern and Southern Italy. The remaining data were split according to their provenance into western fringe of Europe, Britain, Iberian Peninsula, Mainland Europe, Eastern Europe (Balkan Range and Eastern Plains), Balkan Peninsula, Anatolia, Middle East and Egypt (Troy et al. 2001; Beja-Pereira et al. 2006). An MDS plot showed the closer position of Tuscany to the Balkan Peninsula, Anatolia and the Middle East than to Northern and Southern Italy, which clustered with northwestern Europe and Britain, instead.

3. RESULTS AND DISCUSSION

We sequenced 271 bp of the HVRI mitochondrial region in 164 B. taurus belonging to 11 Italian breeds, and we found 61 different haplotypes defined by 47 polymorphic sites. According to the current nomenclature (Troy et al. 2001; Mannen et al. 2004), mtDNA haplotypes can be subdivided into five major haplogroups: T; T1; T2; T3; and T4. With the exception of the Eastern Asian T4 clade, all the other haplogroups were observed in the Italian samples.

Italian bovine sequences were then compared with HVRI sequences from all over Europe, Anatolia, Middle East and Egypt. Reynolds distances calculated at breed level are displayed through MDS in figure 1a, in which an approximate southeast–northwest geographical gradient is recognizable.
The mean numbers of pairwise nucleotide differences between haplotypes were respectively 1.71 (s.d. = 1.01) in cattle from Northern Italy and 1.52 (s.d. = 0.93) in Southern Italy, consistent with the values found in Mainland Europe (1.77; s.d. = 1.04). A significantly higher value of 3.63 (s.d. = 1.87) was observed in Tuscany, comparable to the values found in the Middle East (3.91; s.d. = 2.01) and Anatolia (3.55; s.d. = 1.83). Student’s t-test with Bonferroni’s correction indicated a significant difference (p ≤ 0.001) in the mean number of pairwise nucleotide differences, when Anatolia and the Middle East were compared with Mainland Europe, Northern and Southern Italy. Conversely, non-significant differences were compared with Mainland Europe, Northern and Southern Italy. Accordingly, non-significant differences between Anatolia, Middle East and Tuscany were found.

Median joining networks of sequences from Tuscany revealed a sudden burst of diversity: the numerically more highly represented T3 haplogroup occurred together with several haplotypes belonging to the T, T1 and T2 haplogroups (figure 2). The presence of these four clades at the same time is a peculiar feature shared only with Anatolia and the Middle East, and Africa (table 1; sensu Troy et al. 2001). Cattle raised in these three geographical areas might be genetically differentiated as a consequence of a single genetic origin in the Fertile Crescent followed by strong drift effects during the Neolithic dispersal, or owing to an independent domestication or a local introgression of Bos primigenius mtDNA. Moreover, these areas may probably have contributed in shaping the gene pool of Italian breeds, for either geographical proximity or historic ties. Admixture analysis showed that almost 60% of the mtDNA composition in Tuscany could be assigned to the Anatolian and Middle Eastern bovines, contrasting with values around 0 estimated for Northern and Southern Italy. On the contrary, for the last two populations, a very large European contribution was revealed.

Two hypotheses may explain the peculiar genetic composition in the mtDNA of Tuscan breeds sea trade or human migration. If local traders imported cattle from Eastern Mediterranean regions, only bovines should carry a clear Eastern molecular signature. On the contrary, if newcomers from the Levant brought over their own domestic animals when settling in Tuscany, then also in the modern inhabitants of that area, should a genetic tie with Eastern populations be recognizable.

Novel findings on humans reveal a genetic contribution from the Near East to the modern Tuscan genetic make up (Achilli et al. in press). This issue is clearly disclosed in the mtDNA-based MDS plot of figure 1c, where Reynolds genetic distances calculated between human populations are represented. The geographical areas were chosen following the same criterion previously adopted for the B. taurus sequence analysis, except for Tuscany whose populations were kept separate. The proximity of modern Tuscans from Casentino and Murlo to Anatolian and Middle Eastern human populations does not fit the genetic landscape of Italy, and may derive from a migratory event subsequent to the Neolithization of the Italian Peninsula. Such a presence of an Eastern genetic signature in humans challenges the cattle trade hypothesis.

Even if a stochastic origin of the genetic discontinuities observed in cattle and humans cannot be completely ruled out, the most parsimonious explanation is a single and contemporaneous arrival of humans and cattle. Considering the persistence of the same cattle breeds in Tuscany at least since the first century AD, as recorded by the Roman writer Columella (De re rustica, VI, 1–2), the migratory event suggested in this paper should pre-date the Roman age.

The event is also unlikely to date back to the Neolithic colonization of Italy (ca 6000 BC), since this process took place gradually from the southern part of the peninsula northward (Malone 2003). Such a pattern does not match the sudden burst of diversity in cattle mtDNA observed in Tuscany.

Interestingly, when the phylogenetic relationships of all B. taurus T3 haplotypes were depicted through a single network (data not shown), the 16 050 transition was found to define a branch with the basal haplotype shared by Tuscany (n = 2), Southern Italy (n = 1), Middle East (n = 1), Anatolia (n = 1) and Egypt (n = 2) and three derivative haplotypes—defined by mutations at positions 16 057, 16 138 and 16 261, respectively—present in Tuscany (n = 1), Eastern Europe (n = 2; the Balkan Range and Eastern Plains) and Egypt (n = 1).

The average number of base substitutions (ρ; Forster et al. 1996) in HVRI (from position 16 042–16 313) within this T3 branch and the associated standard error (σ) calculated in the manner of Saillard et al. (2000) were 0.36 ± 0.22. This corresponds to a coalescence time of 3970 ± 2400 years BP using the evolutionary rate estimate of one base substitution per 10 928 years (Troy et al. 2001; Reynolds et al. in press).
Thus, the arrival in Tuscany of the 16,050 motifs should be subsequent to the Neolithic colonization of the Italian Peninsula.

Archaeological remains from Italy favour a relative cultural continuum until the Recent Bronze Age (thirteenth to twelfth century BC). At the birth of Proto-Italians (thirteenth to tenth century BC), a significant break is registered in archaeological data, since some new phenomena appeared in Central Italy, namely the replacement of inhumation by cremation, the spreading of biconical urns and other artefacts and the onset of proto-urban settlements, initially in southern Etruria, followed by an impressive demographic concentration (van der Meer 2004). Later, during the Iron Age (ninth to eighth century BC), in the same areas where the Proto-Italians dwelt, the proper Etruscan culture arose.

We hypothesize the final Bronze Age cultural gap to be related to the arrival in Central Italy of Eastern settlers. These people, together with their cattle, sailed and docked to Tuscany, perhaps forced by the consequences of some catastrophic environmental events like the earthquake storms occurred in the Late Bronze Age in the Aegean and Eastern Mediterranean (Nur & Cline 2000). The mingling of such people and animals with autochthonousItalic populations and livestock probably sowed the seed of Etruscan culture and also shaped the genome of local cattle breeds.

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