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RESEARCH ARTICLE

An independent event of Neolithic cattle domestication on the South-eastern Balkans: evidence from prehistoric aurochs and cattle populations

Peter Hristov⁎, Nikolai Spassovb, Nikolai Ilievb and Georgi Radoslavova

⁎Department of Animal Diversity and Resources, Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences, Sofia 1113, Bulgaria; bPalaeontology and Mineralogy Department, National Museum of Natural History, Bulgarian Academy of Sciences, 1 "Tsar Osvoboditel" Blvd, Sofia 1000, Bulgaria

ABSTRACT

Neolithic/Chalcolithic livestock domestication is an important issue for understanding the mode of life and economics of ancient human communities. The Balkans appears to be a crucial point for clarifying the socio-economical interrelations between the Oldest Middle Eastern/Anatolian and newly formed cultures in Europe. Two main hypotheses regarding the early history of cattle domestication, from their ancestor – the aurochs, have been discussed: multipoint domestication centers or single point origin and subsequent worldwide dissemination. In this study, we provide molecular data about the Balkan aurochs for the first time as well as additional information for the Neolithic/Chalcolithic cattle populations in this geographic location. A total of seventeen samples from different ancient settlements were analyzed according to D-loop control region. The results did not show different genetic profile of wild and domestic populations. All haplotypes were found to belong to the basic macro-haplogroup T. The majority of specimens (n = 14) were defined to form a new Balkan-specific T6 haplogroup. Only two of the ancient samples analyzed were assigned to the T3 haplotype predominating in Europe. We attempt to throw new light on the earliest cattle domestication events in Europe, thus, the results presented are discussed in two directions: (a) The possibility of local independent domestication processes in Neolithic South-Eastern Europe; (b) The single point domestication in the Middle East and subsequent cattle dissemination in Europe. Our data does not exclude the possibility for independent domestication events followed by a second wave of parallel dissemination of cattle herds via the Mediterranean route.

Introduction

Ancient Balkan settlements

The Balkans were defined as the earliest area of European civilization. In this region, one of the earliest pottery Neolithic and Chalcolithic cultures occurred (6000–4000 BC) and formed the so-called “climax period” (Chapman, 2014). Some of the representative cultures from these prehistoric periods as Vinča, Varna, Karanovo, Hamangia were known under the names of the settlements where they were found (Bailey, 2000; Chapman, 2013; Slavchev, 2004–2005). Close geographic relations with Middle Eastern cultures from Fertile Crescent, especially Anatolian, provided an opportunity for direct transfer of knowledge and trading (including crops and livestock). The features specific for this ancient civilization include the social structure integration and differentiations, improved farming techniques, economics based on the utilization of new materials like colored linear ceramic, first copper and gold metal processing, salt manufactures, etc. (Hiller & Nikolov, 2000; Kreuz et al., 2005). The economical structure of these societies was a premise for the development of religion, social differentiation and cultural achievements including the first linear late Neolithic proto-writing known as the Vinča–Gradeshniza–Cucuteni signs and symbols (Chapman, 2014; Bailey, 2000; Haarmann, 2012; Merlini, 2005).

Cattle domestication: single or multiple events

Regarding to this aspect livestock domestication events are an interesting issue and criterion for economical and technologic progress of human societies. The cattle (Bos taurus) as well as its predecessor, the aurochs (Bos primigenius) are the most studied domestic animals of prehistoric times. On the basis of ancient DNA and present day taurine population analyses (based mainly on mitochondrial D-loop control region) two most probable hypotheses have been discussed: I. Single domestication point from Fertile Crescent (Edwards et al., 2007; Lenstra et al., 2014; Troy et al., 2001) or II. Local domestication events during Neolithic era (Beja-Pereira et al., 2006; Bonfiglio et al., 2010). Recent mtDNA data analysis reveals several basic haplogroups with different local geographical distributions and frequencies (Lenstra et al., 2014). The macro-haplogroup T
includes five haplogroups T1–T5, which are found in all modern cattle populations (Magee et al., 2014). T3 haplogroup is predominant among European taurine populations, although it is probably of Middle East origin. The T5 is one of the specific haplogroup but it is rarely disseminated in Europe and reported only in Italy (Bonfiglio et al., 2010) and Croatia (Ivankovic et al., 2007). In contrast to T5, the evolutionary closest to macro-haplogroup T, haplogroup Q is found both in ancient Neolithic and modern cattle (Bonfiglio et al., 2010; Edwards et al., 2007). The haplogroup Q is represented broadly in Europe, though very low frequencies and with possible parallel dispersal along with T3 haplogroup from Middle East (Bonfiglio et al., 2010). Recent data on the ancient cattle population (from Neolithic to Bronze ages) have shown predominant presence of Q haplogroup up to 50% in Iran (7000–5000 BC) as well as in South-Eastern Europe (the Balkans, 6200–2200 BC) (Scheu et al., 2015). The same research has pointed out the trace of Q haplogroup in other part of Europe, even in Anatolian region. The authors justified the obtained results with parallel dissemination along with T3 haplogroup from Fertile Crescent. The same study does not explain why Q haplogroup disappeared in ancient cattle population from Central and West Europe and what is the reason for the same haplogroup to survive only in trace frequencies in modern cattle.

Middle East origin of other basic haplogroups T1 and T2 have also been pointed out (Lenstra et al., 2014; Troy et al., 2001). The T1 haplogroup is disseminated predominantly in Africa and South Europe (Bonfiglio et al., 2012). The T2 has been found only in few exclusive South European and Asian cattle populations (Lenstra et al., 2014). Recent data from ancient Neolithic/Chalcolithic Iberian cattle population have pointed out that T1 haplogroup already exists simultaneously in South-Western Europe (Colominas et al., 2015). Up to date there are no data for the presence of T1 haplogroup in ancient South-Eastern Europe.

Finally, the T4 haplogroup is specific for North and East Asia and perhaps represents cattle domestication from local aurochs population (Mannen et al., 2004). Ancient aurochs mtDNA analysis from Central, West Europe and South Europe (Italy) revealed three main aurochs haplogroups, named P, E and R, which are “clearly” evolutionary distinct from macro-haplogroup T. Among them, only R haplogroup has survived to date in some Italian cattle breeds (Bonfiglio et al., 2010). This study provides additional information as concerning the South-Eastern Balkans aurochs population as well as Neolithic/Chalcolithic cattle domestication events.

Materials and methods

Archeological material collection

A total of 17 ancient samples (8 – B. primigenius; 7 – B. taurus and 2 – B. taurus/B. primigenius (B.t./B.p.) were collected from seven archeological sites in Bulgaria. The samples cover both early and late Neolithic period (n = 12), Early and Late Chalcolithic (n = 3), Bronze Age (n = 1) as well as Medieval Ages (n = 1). More details on archeological sites and excavations as well as on the age of the material are given in Figure 1 and Supplementary Tables S1 and S2. Archeological samples were taken from subfossil bone archaeozoological material, stored in the collections of the National Museum of Natural History, Sofia, at the Bulgarian Academy of Sciences (NMNH). The morphologic assessment of skeleton material was performed in the same institution based on morphological and morphometric criteria (Supplementary Table S2) (Bökönyi, 1995; Iliev, 1994; Tsalkin, 1965; Vasilev, 1985) permitted for reliable classification of the bone material to the aurochs or to the domestic cattle. Bone nomenclature and measurements were based on international standards for archaeozoology (Barone, 1995; von den Driesch, 1976).

aDNA isolation

DNA was extracted from bones and teeth of 17 ancient samples based on the protocol described by Yang et al. (1998) with a minor modification. Initially, to prevent contamination of bone surface the samples were treated subsequently with bleach, ethanol and finally washed with ultra pure water. Bone powders were generated by drilling the bone surface (~500 μg). Bone powder was ground manually with ceramic pestle, dissolved in 5 mL extraction buffer (0.5 M EDTA, 2% sodium dodecyl sulfate, 10 mg/ml dithiothreitol (DTT), 1 M Tris, pH 8.0 and 100 μg/mL proteinase K) and incubated in a rolling thermostat at 55°C for 48 hours. The extraction solution was centrifuged at 5000g for 1 hour; the supernatant was filtered using 0.4 μm filters and transferred to 50 mL tubes. DNA extraction was performed by silica based membrane technology using standard GeneJET™ Genomic DNA Purification kit (#K0721 Thermo-Thermo Fisher Scientific Waltham, MA USA). The GeneJET™ PCR Purification kit column (#K0701 Thermo-Thermo Fisher Scientific Waltham, MA USA) was used to enhance DNA yield of fragments between 100 and 10 kb. The initial steps of bone powder collection were performed in a laboratory localized at NMNH (Sofia, Bulgaria), while the DNA extraction took place at Molecular Evolutionary Laboratory at the Institute of Biodiversity and Ecosystem Research (Sofia, Bulgaria). All experiments were performed according to the standard precautions for good practice for working with aDNA (Paabo et al., 2004; Willerslev & Cooper, 2005).

PCR amplification and sequencing

Three different overlapping segments were used to amplify D-loop control region I. 15 737–16 031 bp (293 bp); II. 15 885–96 bp (548 bp) and III. 16 962–539 bp (893 bp). These positions were alligned to Bos taurus reference sequence V00654 (Anderson et al., 1982). Nested PCR strategy with internal PCR amplification and sequencing oligonucleotide was applied to obtain better and more reproducible results for amplification of Ithl and Ithl segments (Supplementary Table S3). Final lengths of Ithl and Ithl amplicons were 15 962–32 bp (407 bp) and 16 237–403 bp (503 bp), respectively. A strategy for amplification of D-loop control region is shown in Supplementary Fig. S1.

All PCR reactions were performed with 10 ng DNA template in 50 μl final volumes under the following conditions: initial denaturation at 94°C for 5 min, 40–60 cycles (denaturation at
94 °C for 30 s, primer annealing at 55 °C for 30 s, extension at 72 °C for 1 min) and final extension at 72 °C for 10 min followed by Nested PCR with internal primers under the same conditions. The amplified products were sequenced in both directions by Eurofins Genomics Ebersberg, Germany.

Sex determination was performed according to Bollongino et al. (2008) with sex-specific primers for the ZFX/Y genes (Supplementary Table S3). In order to verify authenticity of the sequences, at least two independent extractions, amplifications and sequencing were carried out for all samples and blank controls were performed with each DNA extraction procedure and PCR reaction.

**Statistical and phylogenetic analysis**

After proper processing, 16 sequences (one sample has failed) were obtained and analyzed using MEGA software version 5.0 124 (Tamura et al., 2011). All samples possessed the Iith (internal) segment. Overlapping fragments were manually aligned and assessed. In order to define mytotype identity, all sequences were aligned to the Bovine Reference Sequence (BRS, Acc. no. V00654, Andersson et al., 1982). Sequences were analyzed by polymorphic SNPs position and haplogroups were determined according to Achilli et al. (2009). Representative sequences of five T haplogroup (T1, T2, T3, T4 and T5), P, R, E and Q (Table 1, Supplementary Table S4) were compared by ClustalW2 software package (Larkin et al., 2007) and BLAST analysis (Altschul et al., 1990). Two ancient sequences from the Balkan region (AP6 (JX870109), AP7 (FJ005305) (Bollongino et al., 2006, 2008) were also included in the analysis. The sequences obtained from the present research were deposited in GenBank under Acc. no. KP682315–KP682330. Phylogenetic relationships of mtDNA haplotypes were explored with a Reduce Median network using NETWORK 4.5.1.6 (Fluxus Technology Ltd.). The Rooted Neighbor-Joining (N–J) phylogenetic tree was performed to analyze relationships between main bovine (T1, T2, T3, T4 and T5) and aurochs haplogroups (P, Q, R, E) (Figure 2). The evolutionary distances were computed using the Maximum Composite Likelihood method and were within the units of the number of base substitutions per site. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (10 000 replicates) is shown next to the branches. All positions containing gaps and missing data were eliminated. Patterns of sequence variation,
<table>
<thead>
<tr>
<th>Haplogroups</th>
<th>GenBank Acc No</th>
<th>Haplotypes</th>
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<tr>
<td>T3</td>
<td>V00654</td>
<td>BRS</td>
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<tr>
<td>Q</td>
<td>FJ971080</td>
<td>Bt Rom445</td>
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<td></td>
<td>JX87019</td>
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<td></td>
<td>FJ005305</td>
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<tr>
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<td>JX870109</td>
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<tr>
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<td>R</td>
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</tr>
<tr>
<td>E</td>
<td>DQ915540</td>
<td>Bp EIL04</td>
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Table 1. The variable positions in D-loop control region sequences of the main auroch haplogroups (P, Q, R and E), novel auroch/cattle (T6) and cattle T3 haplogroups with a reference sequence GenBank Acc no V00654.
number of variable sites, mitochondrial haplotype diversity (h) and nucleotide diversity (θ) were estimated, all measures were calculated using DnaSP Ver 5.10.01 DnaSP 5.10 software (Librado & Rozas, 2009).

Results

Data summary

Of all 17 ancient skeleton materials, 16 were successfully amplified, sequenced and included in the analysis. Among them, 8 were morphologically identified to belong to aurochs species (B. primigenius), 6 referred to cattle (B. taurus) and 2 samples with isolation source teeth were defined as B.t./B.p. Analysis of all sequences, both aurochs and cattle showed that they belonged to the macro-haplogroup T (Table 1). The sequences with Acc. no. KP682315–KP682317 contained the full D-loop control region. The sequences KP682318, KP682321, KP682323–KP682326, KP682328 and KP682330 covered only the Ilh and the Ilth region. Thus, most of the haplotypes turned out to be unique and had to be analyzed individually. The analysis also used two additional sequences (AP6 and AP7) from Asagi Pinar (Turkey) due to their geographic locality, especially their Neolithic origin (Bonfiglio et al., 2006, 2008).

Sex determination according to ZFX/ZFY genes generated 14 successful amplifications (8 male and 6 female individuals). The genotypic data were assigned to morphological measurement. The obtained results showed prevalence of male aurochs (75% 6/8), while the taurus sets are presented by only one bull (20% 1/5). Two undefined B.t./B.p. individuals (molar remains) were males (Supplementary Table S1).

T3 haplogroup

Only two samples revealed basic T3 haplogroup. The first one belonged to aurochs (DS1, Chalcolithic) and the second was referred to B.t./B.p (MP23, Neolithic). There was an additional sequence AP6 (cattle) belonging to T3 haplogroup from the Neolithic Balkans (Bonfiglio et al., 2006). Thus, all T3 samples represented only (17%, 3/18) from different settlements and are found both in aurochs and cattle. From T3 haplogroup, two individuals showed male genotype (Suppl. Table S1).

T6 haplogroup

Predominant haplotypes (78%, 14/18) from all samples shared characteristic SNPs motif 16057A-16133C. Seven of them belonged to aurochs (MP341, MP342, MP344, ChM32, Bal343, Pre33 and DC31), 6 were from cattle (MP2, MP21, MP22, MP24, Yba12 and Yba13) and one (MP1) was from B.t./B.p. Regarding both aDNA and modern samples survey in GenBank data sets, we did not find sequences with such kind of motif, except data from two independent investigations (Kantanen et al., 2009, Acc. no. FJ014438-FJ014440; Hristov et al., 2015b, Acc. no. KF373028). Surprisingly, all deposited sequences in GeneBank up to date with motif 16057A-16133C were located only on the Balkans. The results from ancient and recent mtDNA samples give us a reason to define a new haplogroup named T6. Most likely, this haplogroup is a branch of T macro-haplogroup (Figure 2). Another specific feature of T6 haplogroup is a transition at position 15978A/T, divided into equal parts of T6 haplogroup.
Discussion

Neolithic and chalcolithic periods: initial steps for understanding ancient human societies and civilization

With regard to the most popular evidences it is considered that only some regions have been discussed as a cradle of civilization regarding social technology, knowledge and revolutionary success. In this context the Fertile Crescent (Middle East, Mesopotamia), Indus valley (today Pakistan) and Nile river valley (Ancient Egypt) were pointed out as regions in which the beginning of civilization took place worldwide (Kriwaczek, 2012; Midant-Reynes, 2000; Ray, 2003). In contrast, the surrounding regions (in particular Europe) were described as beneficiaries of knowledge and trades. In this light, the domestication of livestock and crops seems to be a reliable and broadly studied criterion for early stage civilizations.

In this paper, we attempted to elucidate the issue of Neolithic/Chalcolithic European cattle domestication. In this regard, two main hypotheses exist: a single point origin from Fertile Crescent (C24 10 500 BP) (Edwards et al., 2007; Lenstra et al., 2014) or multipoint independent local domestication in different geographical localities (Bonfiglio et al., 2010). The spreading of livestock from a domestication center into Europe, Africa and the rest of Asia is a key moment for the understanding of this issue.

European aurochs populations

Most of the studies (mainly from West/Central Europe) showed that European aurochs populations were undoubtedly different (based on mtDNA data) from Middle East domesticated cattle. While haplogroup E in Germany was with local distribution, the most widely distributed among European aurochs was haplogroup P (Edwards et al., 2010) and R in Italy (Bonfiglio et al., 2010). In contrast, the main characteristics of Middle East aurochs population as well as cattle descending from it was macro-haplogroup T, especially T1, T2 and T3 (Lenstra et al., 2014). There were no molecular data for aurochs populations from East and South-Eastern Europe, although they appeared to be a border area in Eurasia. Here we have reported the first molecular data from aurochs populations from the South-Eastern Balkans. The results showed that all aurochs (n = 8) belonged to macro-haplogroup T, they share the common T3 (n = 2) and the newly described branch of macro-haplogroup T, named T6 (n = 7) (Figures 1 and 3).

Haplogroup T3

The presence of T3 haplogroup among Balkan aurochs populations is not surprising for this region, because of its proximity to the Anatolian population, due to intercontinental terrestrial root during Last Glacial Period up to Early Neolithic ~5600 BC (Black Sea deluge hypothesis, Eris et al., 2008). More interesting was the simultaneous presence of aurochs T3 haplogroup on the Italian peninsula (Beja-Pereira et al., 2006; Achilli et al., 2009). This group even predominated over the Italian aurochs population, which was a clear evidence for coexistence of T3 haplogroup in South Europe and the Balkans (Figure 1).

Q haplogroup – an enigmatic case of its appearance and disappearance in ancient and modern cattle populations

The Q haplogroup is described for the first time by Achilli et al., (2008). Till date three complete mitochondrions defined as Q haplogroup (Achill et al., 2008) and eight modern cattle partial D-loop sequences (motif 15953G-16255C) exist in GeneBank data sets (three entries Acc.no. DQ166107, AY495552, EY281475, Jia et al., 2010 from China; one Acc. no. EF126311
from Turkey (Anatolia), Ozdemir and Dogru 2009; one Acc. no. FG815451 from Portugal, Ginja et al., 2009 and additional three deposited sequences from Italy – Acc. no. EY177866 (Achilli et al., 2008) and Acc. no. HQ184030, HQ184039 (Bonfiglio et al., 2010). Moreover, there are 16 undeposited in GenBank sequences from Italy, which represented about 1% (16/1747) animals (Bonfiglio et al. 2010). These data characterized Q haplogroup as sporadically represented group worldwide, and this seems to be more specific only for Italian present day cattle breeds.

This haplogroup also was defined with sporadic frequency in ancient cattle samples: four from Fertile Crescent (Iran; Bollongino et al., 2012; Lenstra et al., 2014) and four samples dispersed throughout ancient Europe (one from Germany (Bollongino et al., 2006); one from France (Edwards et al., 2007); one from the European part of Turkey (Bollongino et al., 2008) and one from Finland (Niemi et al., 2015). Interesting and unexpected results for high frequency of haplogroup Q (up to 50%) in ancient cattle population are shown in the paper of Scheu et al. (2015). The authors have found this haplogroup in Neolithic and post Neolithic ages (Fertile Crescent) up to 40%, but Q haplogroup is not found in Western Anatolian. The ancient cattle samples from Europe show that Q haplogroup predominated in South-Eastern Europe (Balkans): from Early Neolithic (6200–5500 BC) to Bronze ages (2700–2200 BC) – 50% and 30%, respectively. Also, in Neolithic Italy (6000–5500 BC) this haplogroup is represented by 20%, while in Central/ Western Europe the Q haplogroup is sporadically represented with only about 4% (Scheu et al., 2015). Altogether, data from ancient cattle populations (Scheu et al., 2015) reveal that Q haplogroup is the second spread after T3, from domesticated center to ancient Europe. The reduction of frequency of Q haplogroup both in ancient and modern cattle may be explained as bottleneck type decreasing. Scheu et al., (2015) give no explanation: I. What is the reason for such kind of change of dissemination in ancient Europe cattle population (Southern-Eastern, Western Europe)? II. What is the reason for losing the Q haplogroup in modern cattle at the place of its origin, especially Middle East and the Balkans? These results are confusing regarding to the basic hypothesis for single point origin from Middle East and subsequent dissemination of ancient cattle to Western Europe.

At first reading our results reveal fully different genetic profile of Balkan ancient cattle. On the other hand, the presence of T6 haplogroup among our data sets, instead of Q haplogroup seems to be confusing. This may be explained only by the different approach concerning mtDNA amplification strategy between the two research groups (Supplementary Fig. S1). Scheu et al., (2015) have used conventional strategy for ancient DNA amplification, based on construction of short overlapping fragments from D-loop region. In contrast to this protocol, our study represents another strategy based on ancient DNA PCR amplification of longer fragments using Nested PCR amplification (Supplementary Fig. S1). This approach improved the effectiveness concerning DNA analysis of sequenced fragments and it is similar to methods used for the analysis of recent cattle population (Hristov et al., 2015a,2015b; Kantanen et al., 2009).

**Newly named T6 haplogroup**

The predominant haplogroup among Balkan aurochs and cattle samples (over 80%) was T6 haplogroup, based on motif 16057A-16133C. This motif was found with high frequency only in present day autochthonous Busha cattle breed from Serbia (Kantanen et al., 2009) and Bulgaria (Hristov et al., 2015a,2015b) and to the present day nowhere else worldwide. This gives us a reason to define this group as a Balkan specific and representative. In fact, the presence of local T6 haplogroup spreading only on the Balkans is not surprising. Additionally, a few separate investigations reported other haplogroups also European specific with regional locality - R and T5 (Achilli et al., 2008, 2009; Ivancovic et al., 2014). The haplogroup R was distributed only in Italy which could be explained by the existence of a refugial population. The other T5 haplogroup is presently identified only in few Italian and Croatian cattle breeds with undefined origin, most likely ancient regional creation.

**Balkan cattle domestication: an origin from local aurochs population or/and a transition point of spreading of early domesticated anatolian cattle via the mediterranean and the danube river routs?**

The first archeological data about cattle domestication indicated that taurine cattle were domesticated between 10.800 and 10.300 years ago in the Fertile Crescent, most probably on the western Turkish-Syrian border (Helmer et al., 2005; Vigne, 2011). In Europe, first data for domestication was ca. 8000 BP (Yabalkovo Neolithic settlement, Bulgaria) (Roedenberg, 2014) from the Balkans which correlated with early Pottery Neolithic age. During this period, the Balkan cattle coexisted with other domesticated animals (dogs, sheep, goats and pigs). The increasing use of domestic cattle in Anatolia and South-Eastern Europe, which was recorded in some cases over 30% Bos remains of total number of identified species (Todorova and Vajsov 1993; Boessneck and von den Driesch 1979; Iliev and Spassov 2007). Neolithic/Chalcolithic human societies exploited the capacity of livestock not only as a major source of milk, meat, and hides, but also for accelerated technological progress, transport and trading. It is well known that domesticated cattle were used to drag sledges and, sometime later, plows and wheeled wagons from about 4000 BC (an almost simultaneous innovation in the Middle East and in Europe) (Sherratt 1986; Bailey 2000; Bellwood et al., 2007).

In contrast to archeological data, molecular analysis both from ancient and modern cattle revealed their origin from Τ macro-haplogroup (T, T1, T2 and T3) wild aurochs (Lenstra et al., 2014). It is well known that all of these haplogroups have Middle Eastern origin. All known European aurochs populations have been identified to carry mtDNA haplotypes that were classified as P or E. At the same time, there is only few data showing the presence of T3 type aurochs in Europe (Beja-Pereira et al., 2006; Achilli et al., 2009). Totally, the most probable hypothesis for cattle domestication seems to be the one of single point origin (Anatolia) and later dissemination of cattle to Neolithic Europe. In this point of view, the Balkans
appear to be a crucial region for supporting or rejecting this hypothesis.

The results presented in this paper showed that all ancient mtDNA data both cattle and aurochs represented non-P, but T haplotypes. Moreover, the T3 haplotypes were unexpectedly rare among Neolithic/Chalcolithic populations, in contrast to predominant Balkan-specific T6 haplogroup (Figures 1 and 3). T6 haplogroup was found only in the Balkans in ancient DNA samples, but it still exists in present day aurochthons brachicerous cattle form Rhodopa mountain (Bulgaria) and Serbia (Hristov et al., 2015a; Kantanen et al., 2009). These data clearly demonstrate a Neolithic origin of these cattle breeds (Ajmone-Marsan et al., 2010). Further, the absence of T6 haplotypes out of the Balkans in the North/West direction do not exclude gene flow between wild and domesticated forms carrying T macrohaplogroup or possible case of independent domestication in Neolithic/Chalcolithic Europe.

Our results clearly demonstrated that animals that carry T3 haplogroup did not disseminate in Europe via Eastern Balkan route (Danube route hypothesis) due to it is did not exist in Neolithic West/Central Europe. So, where did the T3 cattle pass from and how did they reach West/Central Europe simultaneously? Until now there is no aDNA data for the West and South Balkans, but the presence of T3 Italian aurochs (coexisting with present day R, Q and possibly T5) does not exclude a possibility of other local domestication centers in Europe. The Anatolian derived T1 and T2 haplogroups were with different frequencies among European cattle populations. Both haplogroups existed only in South Europe (Mediterranean region and Balkans) of about 10% presence. Ancient Iberian cattle samples indicate that T1 haplogroup still coexists with T3 from Neolithic/Chalcolithic ages (South-Western Europe) (Colominas et al., 2015). The absence of T1 haplogroup in the present research as well as the study by Scheu et al., (2015), Lenstra et al., (2014) indicated that cattle carrying T1 haplogroup did not arrive in Neolithic South-Western Europe via Mediterranean route, but it is disseminated/introduced either North African or local domestication taurine. This hypothesis does not contradict the spreading of animals via Mediterranean route, but more additional data is needed to resolve this issue. The hypothesis of multiple cattle domestication in ancient Europe is possible and plausible and does not exclude, because of the presence of the aurochs T3 type haplogroup found in South Europe and due to the availability of specific European haplotypes both in ancient and modern cattle populations.

**Conclusion**

The molecular analysis of Balkan (Bulgarian) cattle and aurochs populations demonstrated identical haplotypes. This fact gives us ground to suggest a gene flow between aurochs and domesticated cattle or process of domestication possibly independent from the Anatolian region in the Balkan region, and presumably parallel to the beginning of the Neolithic Epoch on the Balkans. It is necessary to note that the size of the early Neolithic cattle population from Yabalkovo settlement, where the absolute calibrated data show an age close to the beginning of the 8th millennium BP, indicates an advanced process of domestication (Spassov & Iliev, 2014). So, the domestication in the region possibly started sometime before this. A process of drawing of local aurochs at the stage of domestication was supposed for the late Neolithic cattle from Gradeshnitsa-Malo Pole, judging by its very large size and the abundance of aurochs remains in the animal bone samples from this prehistoric site (Iliev & Spassov, 2007). The result obtained in this investigation molecular data could change the traditional viewpoint that, all the domestication processes originated in the prehistoric Anatolian region from which all earliest European cattle originate.

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**Declaration of interest**

The authors declare no conflict of any financial and personal relationships with other people or organizations that could influence their work interest.

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