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Maternal variability of Croatian Spotted goat (*Capra hircus*)

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Abstract: This study provides the first characterization of maternal ancestry and mitochondrial DNA (mtDNA) diversity in the Croatian Spotted goat (CSG), the most important autochthonous goat breed in Croatia. CSG (n = 25) were randomly sampled from seven herds and a 660-bp fragment from the mtDNA D-loop region was sequenced. Those sequences were compared with 122 corresponding GenBank sequences from goat populations in Albania, Austria, Egypt, Greece, Italy, Romania and Switzerland. CSG showed a great polymorphism (only three out of 17 haplotypes were shared) with high a haplotype (Hd = 0.967 ± 0.019) and nucleotide diversity (π = 0.01305 ± 0.00068). When compared with Mediterranean and ancient goats, all of the 25 CSG were randomly scattered inside haplogroup A showing the weak phylogeographic structure with within-breed variance accounting for 91.76% of the genetic variation. In addition, population expansion tests (mismatch distribution and Fu's *Fs* statistic) supported these results suggesting at least one population expansion.

Keywords: ancestral maternal diversity; D-loop; Mediterranean goat population; phylogeography; unique haplotypes

The domestic goat *Capra hircus* is one of the first domesticated animal species. Goat domestication occurred around 10 000 years ago from the wild bezoar in the Fertile Crescent (Zeder and Hesse 2000). From there it spread to Europe by two main routes: the Danubian and the Mediterranean route (Diamond and Bellwood 2003). Archaeological evidence suggests that goats inhabited the Croatian territory during the Neolithization process (Radic 2018). Today in Croatia there are three autochthonous goat breeds out of which the Croatian Spotted goat (CSG), with 1435 head, represents 85% of the breed composition. The other two breeds are Croatian white goat with 229 and Istrian goat with 36 animals.

Genetic diversity of animals is analysed using different markers such as mitochondrial DNA, Y chromosome, microsatellite or single nucleotide polymorphism (SNP). Molecular studies based on mitochondrial DNA (mtDNA) sequences have been used to determine the origin and phylogenetic diversity of goats (Luikart et al. 2001; Sultana et al. 2003; Amills et al. 2004; Joshi et al. 2004; Chen et al. 2005; Liu et al. 2006; Sardina et al. 2006; Naderi et al. 2007; Amills et al. 2009; Vacca et al. 2010; Lin et al. 2013). Since goats show a weak geographical structure and extensive intercontinental gene flow (Luikart et al. 2001), mitochondrial DNA seems to be the most appropriate genetic tool for the first insight into goat phylogenetic diversity (Brown et al. 1986). The phylogenetic analysis of goat mtDNA has revealed six divergent lineages in the world – A, B, C, D, F and G (Luikart et al. 2001; Sultana et al. 2003; Joshi et al. 2004; Chen et al. 2005; Sardina et al. 2006; Naderi et al. 2007). Three lineages (A, C and F) have been detected in 3 countries neighbouring Croatia: Italy (Sardina et al. 2006), Slovenia and Albania (Naderi et al. 2007).

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The aim of this study was to analyse a 660 bp mtDNA D-loop fragment of the CSG, as well as to contribute to the characterization of the domestic goat by comparing the Croatian autochthonous goat with goat populations from neighbouring countries: Italy, Albania, Romania, Switzerland, Greece, Austria and Egypt.

MATERIAL AND METHODS

Blood sampling and data sets. Blood samples were taken from 30 CSG randomly sampled on seven farms in southern Croatia representing both sexes (16 females and nine males). Our sampling covered 65% of the entire known CSG breeding area. As a result of DNA isolation and sequencing errors five samples were lost and further analyses were done on 25 samples. The CSG is reared in extensive systems with the total population of just over a thousand animals. This dataset accounts for 1.5% of the CSG population, which is assumed as representative. Blood from *v. jugularis* was collected into EDTA test tubes and stored at 4°C until DNA extraction.

Sequence analysis was based on several datasets including results from this study. For comparative sequence analysis, we retrieved goat mtDNA sequences autochthonous to Italy (61), Albania (13), Austria (6), Greece (11), Slovenia (5) and Switzerland (26) from GenBank. To construct a neighbour-joining tree, 28 reference mtDNA haplotypes were retrieved. For calculating the AMOVA statistic and Reynolds' distance matrix, as well as for constructing a median-joining network, we selected the most frequent haplotypes in 26 goat breeds from 8 different countries (according to a possibility to align in MEGA X software) (Kumar et al. 2018), most of them from the Mediterranean part of Europe. Ancient goat mitochondrial genomes (85 samples) from different archaeological sites in eastern, western and southern regions around the Fertile Crescent were retrieved and compared with recent Mediterranean goat populations.

DNA extraction and mtDNA amplification and sequencing. DNA was extracted from 200 µl of whole blood using the Sigma Gen Elute^{TM} Blood Genomic DNA kit (Sigma-Aldrich Inc., USA). PCR was used to amplify a 660 bp mtDNA control region fragment; thermal cycling conditions and primers were retrieved from Amills et al. (2004) (forward primer: 5'-CGC TCG CCT ACA CAC AAA TA-3'; reverse primer: 5'-AAT GCC CAT GCC TAC CAT TA-3'). PCR products were separated by electrophoresis on 1% agarose gel (Sub-cell, GT, Bio-Rad, Germany) and purified using the Wizard® SV Gel and the PCR Clean-up System (Promega, USA). Sequencing was performed on an ABI PRISM® 3100-Avant Genetic Analyzer (Applied Biosystems, USA) using the ABI Prism BigDye Terminator v3.1 Cycle Sequencing kit. The same primers were used for PCR amplification and sequencing on both strands.

Data analysis. Sequences were aligned using ClustalW (Larkin et al. 2007) and Clustal Omega (Goujon et al. 2010) software for large datasets. Neighbour-joining (NJ) trees were constructed using MEGA X software (Kumar et al. 2018). A median-joining network was drawn using Network software version 5 (Bandelt et al. 1999). Statistical data were analysed using Arlequin software version 3.5 (Excoffier and Lischer 2010) that was also used to compute Reynolds' distance. Principal component analysis (PCA) based on Reynolds' distance matrix was conducted using R software version 3.4.1. (R Core Team 2017), package pca3d (Weiner 2017). To analyse the phylogenetic relationships the sequences were aligned using Clustal Omega software to generate a nexus file (Goujon et al. 2010). Bayesian phylogeny was calculated using the BEAST version 1.4.3 software package (Drummond et al. 2012). For the tree calibration, 48 complete mitogenome sequences of 41 goats, 7 sheep, 5 ancient samples of *Bison priscus*, 2 samples of *Bison bison*, 36 head of cattle and 2 ancient aurochs were used. Two different priors were set – I: 6000 ky tree root according to the age of separation between sheep and goat by bone morphometry, and II: the molecular clock that was previously estimated for cattle settled at 4.66×10^{-8}. The program was run with Markov chain Monte Carlo 700 000 000 states length until effective sample size values were over 200. Calculations were performed using two different substitution models: Generalised Time Reversible (GTR) using the relaxed lognormal molecular clock (Lanave et al. 1984) and Coalescent Bayesian Skyline model. To analyse the results, Tracer software version 1.7 (Rambaut et al. 2018) was used. The selected tree file was compiled in TreeAnnotator software version 2.8.7 from the BEAST package. A total of 817 goat reference sequences (372 bp D-loop region) were aligned in

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MEGA X to construct the nexus file. Using GTR, relaxed lognormal clock and Bayesian skyline with the tree root prior for goats set at 139991y, BEAST version 1.4.3., were run. The selected tree file was compiled in TreeAnnotator version 2.8.7 from the BEAST package. The most common ancestor (MCA) trees were constructed in FigTree version 1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/).

RESULTS

Sequence polymorphism. In the sample of 25 CSG, over the 660 bp sequence, 50 polymorphic sites (21 singleton variable sites and 29 parsimony informative sites) and 17 haplotypes were identified (Table 1). Overall, the control region sequenced in this study was highly polymorphic, showing a haplotype diversity (Hd) of 0.967 (standard deviation (SD) 0.019) and a nucleotide diversity (π) of 0.01305 (SD 0.00068).

Phylogenetic relationship. Neighbour-joining tree and median-joining network (Supplementary Figure S1 and Figure S2 in Supplementary Online Material (SOM)) constructed for 25 CSG indicate that they all clustered inside haplogroup A. The median-joining network revealed the most frequent haplotypes among 26 goat breeds in seven countries and showed the intermingling of breeds from different geographic regions (Figure 1). However, haplotypes within the same country did not cluster together.

Population genetic structure. To further investigate the geographical structure, we performed several analyses of molecular variance (AMOVA) at different hierarchical levels. AMOVA parameters for goat breeds analysed in the present study are shown in Supplementary Table S1 in SOM. Global AMOVA suggested that 91.76% of variation was within populations ($P < 0.001$) and only 8.24% between populations ($P < 0.001$), indicating the absence of geographical structuring. The possibility of population expansion was assessed using two approaches, Fu's *Fs* statistic (Fu 1997) and mismatch distribution (Rogers and Harpending 1992). The *Fs* value was negative (–4.40311, ($P < 0.05$)), suggesting recent population expansion. The mismatch distribution analysis revealed one major peak consistent with the negative *Fs* statistic (Supplementary Figure S3 in SOM). In order to illustrate the relationship between the CSG and other Mediterranean goat breeds, the PCA plot was made. It showed that CSG clustered the closest to autochthonous goats of Italy (Maltese, Valdostana, Bionda dell' Adamello, Giorgia Molisana and Girgentana, Argetata dell' Etna), Switzerland (Girson Striped), Albania (Capore, Hasi, Mati) and Greece (Greece Goat) (Figure 2). Overviewing the calibrated phylogenetic tree that represents goat breeds neighbouring Croatia we can see that the CSG is represented throughout the whole haplogroup A and that the calibrated tree confirms the PCA results. The MCA tree for calibration, using tip dates and outlier groups, was

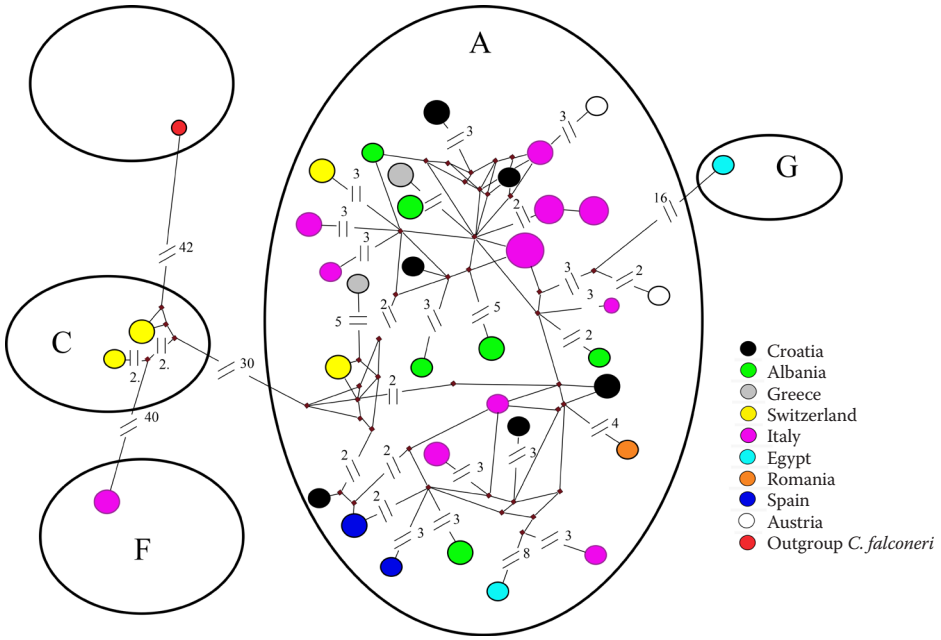

Figure 1. Median joining network of the most frequent haplotypes (480 bp sequences) in 33 goat breeds located in 7 countries neighbouring Croatia. Wild goat *C. falconeri* was used as an out-group. Circle area is proportional to haplotype frequency

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Table 1. Distribution of 50 polymorphic sites (660 bp sequences) found in 17 Croatian Spotted goats haplotypes. The reference sequence GenBank accession No. is NC_005044

	15747	15761	15771	15807	15811	15843	15873	15893	15896	15898	15909	15911	15913	15921	15921	15930	15933	15947	15950	15965	15969	15972	15974	15975	15976
NC_005044	G	C	G	C	T	A	A	T	C	G	A	A	A	A	T	C	A	C	C	C	C	T	T	C	C
Haplotype 1	A	.	.	T	C	G	.	C	T	.	.	.	G	.	.	T	.	T	.	T	.	C	C	T	T
Haplotype 2	A	.	.	T	C	G	.	C	.	.	.	.	G	G	.	T	.	T	T	T	T	.	.	.	T
Haplotype 3	A	.	.	T	C	G	.	C	.	.	.	.	G	G	.	.	.	T	T	T	.	.	C	T	T
Haplotype 4	A	.	.	T	C	G	.	C	.	.	.	.	G	G	.	.	.	T	T	T	T	.	.	.	T
Haplotype 5	A	.	A	T	C	G	.	C	.	A	.	.	G	G	.	T	.	T	T	T	.	.	C	T	T
Haplotype 6	A	.	.	T	C	G	.	C	.	.	.	.	G	.	.	T	.	T	T	T	T	.	C	T	T
Haplotype 7	A	.	.	T	C	G	.	C	.	.	.	.	G	.	.	.	.	T	T	T	T	.	C	.	T
Haplotype 8	A	.	.	T	C	G	.	C	.	.	.	.	G	.	.	T	.	T	T	T	.	.	.	T	T
Haplotype 9	A	A	.	T	C	G	.	C	.	.	.	.	G	G	.	T	.	T	T	T	.	.	.	T	T
Haplotype 10	A	.	.	T	C	G	.	C	.	.	.	.	G	G	.	.	.	T	T	T	T	.	C	.	T
Haplotype 11	A	.	.	T	C	G	.	C	.	.	.	.	G	G	.	T	.	T	T	T	T	.	C	T	T
Haplotype 12	A	.	.	T	C	G	.	C	T	.	.	.	G	.	.	T	.	T	T	T	T	.	C	.	T
Haplotype 13	A	.	.	T	C	G	G	C	.	.	.	.	G	.	.	T	.	T	T	T	T	.	.	.	T
Haplotype 14	A	.	.	T	C	G	.	C	.	.	G	.	G	.	.	T	G	T	.	T	.	.	C	T	T
Haplotype 15	A	.	.	T	.	G	G	C	.	.	.	.	G	.	C	T	.	T	T	T	T	C	C	T	T
Haplotype 16	A	.	.	T	C	G	.	C	.	.	.	.	.	.	.	T	.	T	.	T	T	.	C	.	T
Haplotype 17	A	.	.	T	C	G	.	C	.	.	.	G	G	.	.	.	.	T	T	T	T	.	C	.	T

	15977	15978	15981	15982	15983	15984	15992	16000	16006	16010	16011	16019	16026	16027	16037	16040	16043	16045	16062	16083	16150	16151	16165	16232	16235
NC_005044	T	T	T	A	G	C	G	T	T	C	C	A	A	A	T	C	T	T	T	C	C	C	T	T	T
Haplotype 1	C	.	C	G	A	.	A	.	C	.	T	G	.	G	C	T	C	C	.	T	.	.	.	C	.
Haplotype 2	C	.	C	G	A	.	A	.	C	T	T	.	.	G	C	T	.	C	.	T	.	.	.	C	.
Haplotype 3	C	.	.	G	.	.	A	.	C	T	T	G	.	G	C	T	C	.	.	T	.	.	.	C	.
Haplotype 4	C	C	.	G	.	.	A	.	C	T	T	G	.	G	C	T	.	C	.	T	.	T	C	C	.
Haplotype 5	C	.	C	G	.	.	A	.	C	T	T	G	.	G	C	T	C	C	.	T	.	.	.	C	.
Haplotype 6	C	.	C	G	A	.	A	.	C	T	T	.	.	G	C	T	.	C	C	.	.	.	.	.	.
Haplotype 7	C	.	.	G	.	.	A	.	C	T	.	G	.	G	C	T	.	C	.	T	.	.	.	C	.
Haplotype 8	C	.	C	G	.	.	.	.	C	T	T	G	.	G	C	T	.	C	.	T	.	.	.	C	.
Haplotype 9	C	.	C	G	.	.	A	.	C	T	T	G	G	G	C	T	C	.	.	T	.	.	.	C	.
Haplotype 10	C	.	.	G	.	.	A	.	C	T	T	G	.	G	C	T	.	C	.	T	T	T	.	C	.
Haplotype 11	C	.	.	G	.	.	A	.	C	T	T	G	.	.	.	T	.	.	.	T	.	.	.	C	.
Haplotype 12	C	.	.	G	.	.	A	C	C	T	T	G	.	G	C	T	.	C	.	T	.	.	.	C	.
Haplotype 13	C	.	.	G	.	.	A	.	C	T	T	G	.	G	C	T	.	.	.	T	.	.	.	C	.
Haplotype 14	C	.	.	G	.	T	A	.	C	T	T	G	.	G	C	T	.	C	.	T	.	.	.	C	.
Haplotype 15	C	.	C	G	.	.	A	.	C	T	T	G	.	G	C	T	.	C	.	T	.	.	.	C	.
Haplotype 16	C	.	.	G	.	.	A	.	C	.	T	G	.	G	C	T	.	C	.	T	.	.	.	C	C
Haplotype 17	C	C	.	G	.	.	A	.	C	T	T	G	.	G	C	T	.	.	.	T	.	.	.	.	.

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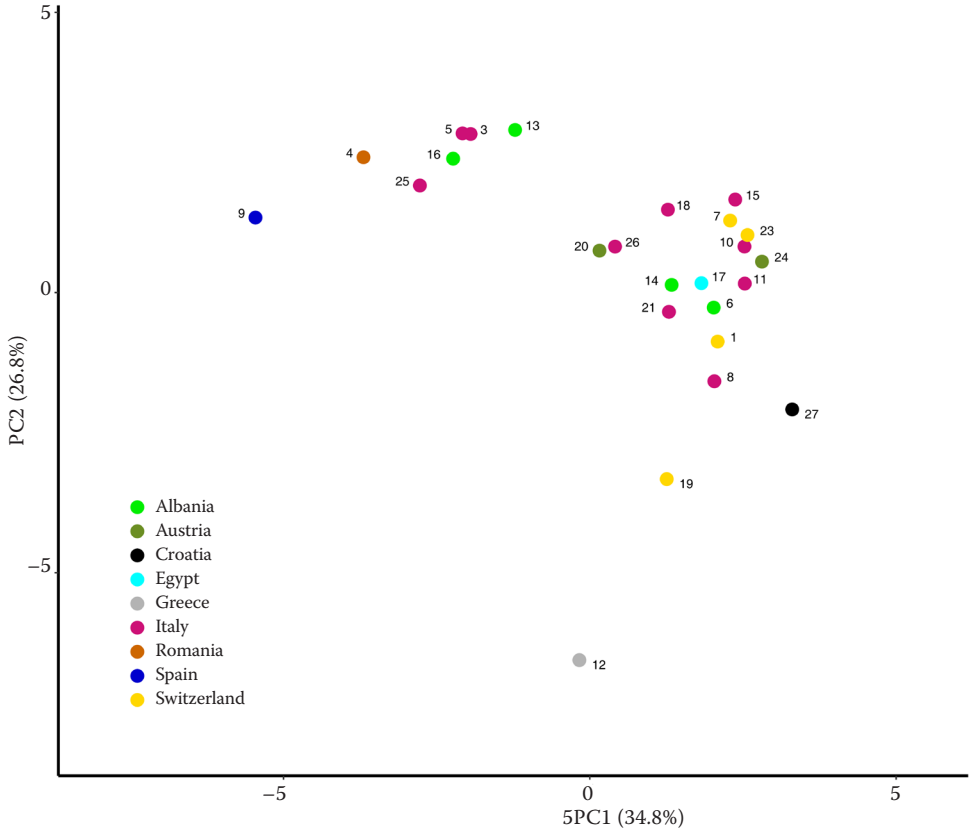

Figure 2. Principal component analysis (PCA) of 27 Mediterranean goat breeds computed using Reynolds' distance matrix (480 bp sequences)

1 = Alpine, 2 = Arbi, 3 = Argetata dell' Etna, 4 = Austria-Romania, 5 = Camoscata, 6 = Capore, 7 = Grison Striped, 8 = Derivata di Siria, 9 = Spanish goat, 10 = Giorgia Molisana, 11 = Girgentana, 12 = Greek goat, 13 = Hasi, 14 = Liqenasi, 15 = Maltese, 16 = Mati, 17 = Baladi, 18 = Orobica, 19 = Peacock, 20 = Pinzgauer, 21 = Sarda, 22 = Skopelos, 23 = St. Galen Botted, 24 = Tauernpied, 25 = Valdostana, 26 = Verata, 27 = Croatian Spotted goat

breed 2 Arbi (x = −10.0351021; y = −2.0368014) and breed 22 Skopelos (x = −2.4906907; y = −8.4121720) are not shown in the PCA plot

calculated with the BEAST 1.4.3. software package (Supplementary Figure S4 in SOM). The MCA tree was calculated using Croatian and Mediterranean recent goat sequences and ancient mitochondrial sequences from Daly et al. (2018) and treeHigh priors from the calibrated tree are presented in Supplementary Figure S5 in SOM.

DISCUSSION

The CSG is the most important Croatian autochthonous goat with no phylogenetical characterization available. We analysed the phylogeographic structure and genetic diversity of a CSG representative sample, and high haplotype diversity, several unique haplotypes and only three shared haplotypes between populations were detected (Figure 1).

Haplotype and nucleotide diversity of mtDNA provides important indices for assessing population polymorphism and genetic differentiation. High values of haplotype and nucleotide diversity in a population indicate high polymorphism (Liu et al. 2006). The very high haplotype diversity obtained for CSG (0.967 ± 0.019) is similar to that of South European and North African goats (Naderi et al. 2007; Martinez et al. 2012). The ratio between the number of haplotypes and animals analysed (0.68) is moderate to low, similar to that of the Sicilian goat population (Sardina et al. 2006). The analysed data showed a high number of unique haplotypes and only three haplotypes were shared between populations (farms). The average number of nucleotide differences between the CSG haplotypes was 9.733, which indicated high diversity like that observed in Maltese and Sarda goat breeds (Vacca et al. 2010).

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The median-joining network analysis of Mediterranean goat haplotypes, including those identified in the CSG breed, showed that all these populations have intermingled. However, haplotypes from each country did not cluster together. These findings are consistent with a weak phylogeographic structure for CSG, as well as for other Mediterranean populations, as previously reported (Amills et al. 2009; Vacca et al. 2010). Following the suggestion of Schnieder and Excoffier (1999), mismatch distribution and Fu's *Fs* were used to seek the population expansion. Negative *Fs* values and mismatch distribution results supported by the low phylogeographic structure observed in highly scattered CSG samples inside haplogroup A suggest that at least one population expansion has occurred in the breed's history.

The tree calculated with the ancient goat sequences suggests that goats have spread to Croatia by the Danubian route. This finding is consistent with the results of Fernandez et al. (2006).

The high variability of haplotypes found in this study showed that even in areas that are very distant from putative centres of domestication there is an important component of diversity. Characterizations of local breeds are very important, allowing a production increase without the loss of local adaptation (Hall and Bradley 1995).

CONCLUSION

The phylogenetic analysis of Croatian Spotted goats suggests that at least one population expansion occurred in the breed's history and this expansion did not prevent the breed from achieving high polymorphism. Unexpectedly, the CSG breed possesses a high number of unique haplotypes for such a small population. The greatest similarity between populations is found with the geographically closest population both in the recent sample comparison as well as in the ancient sample. In a future analysis of the phylogenetic diversity of Croatian goat populations high-throughput markers will be used. In the attempt to reconstruct the Croatian goat spreading route from the domestication centre and the development of the breed throughout history, ancient goat samples from Croatia should be used.

All Croatian Spotted goat sequence data were submitted to the public sequence database (GenBank) under Accession No. JX112296–JX112320.

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