Mitochondrial DNA Diversity in Wild Boars from the Istria and Cres Island

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Summary

The aim of study was to analyze the nucleotide diversity of mitochondrial DNA control region (CR–mtDNA) of 33 wild boars hunted in Istria and Cres Island (Croatia) in order to prove the presence of clade E2 haplotypes outside the Italian Peninsula and Sardinia. Outside of Italy haplotypes from clade E2 have been found only in Istria (Pupcina Cave) on archaeological samples dating to 9000 years BC. Among 21 haplotypes found on a 428–bp sequence fragment representing European wild boars, with 227 sequences additionally retrieved from the GeneBank, our wild boars were assigned in two haplotypes classified in clade E1. Two wild boars, from Oprtalj and Grožnjan, were distributed in the haplotype H2 while the other 31 samples were distributed in the haplotype H6. On the analyzed fragment, observed haplotypes were two (T→C at 15714 and 15758) and one (T→C at 15758) transversions remote from the reference sequence reported in the GenBank under accession number AJ002189 (Ursing and Arnason, 1998). The H2 and H6 haplotypes were both previously found in European wild boars, most frequently in haplotypes from Portugal and Spain. In conclusion, the results obtained were concordant to the hypothesis that clade E2 haplotypes are indigenous to Italy and Sardinia. According to the maternal origin, the Istrian and Cres Island wild boars are close to the Gorizia region (north–east Italy) population as well as to wild boars from Portugal and Spain.

Key words

wild boar, mitochondrial DNA, nucleotide diversity, Istria region, Cres Island

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Aim

Wild boar (*Sus scrofa* L.) is an important game animal widely distributed all over the world. While native to Asia, Europe and North Africa the highest number of wild pig taxa are observed in south–eastern Asia which may be a possible place of its origin. Unlike a majority of domestic species where domestication is strongly linked to the Near East, identifying of the wild boar is far from being resolved. According to Larson et al. (2005, 2007) at least six independent centres have been proposed, among which one or several were linked to Europe. Variability of the mitochondrial DNA (mtDNA), particularly its control region (CR), has been a widely used tool in studying origin of domestication (Giuffra et al., 2000; Kim et al., 2002; Larson et al., 2005). With respect to CR–mtDNA sequence variation there are several clades with geographically specific distributions in wild and domestic pigs, among which well known are clade A found in Asia, clade E1 wildly spread in Europe and clade E2 found only in Italy. Although, there is a vast literature on the distribution of CR–mtDNA sequence variation in European wild and domestic pig populations, including analyses on archaeological samples, there are still a number of gaps that remain unfulfilled. In a review paper, Scandura et al. (2011) pointed to needs for wider sampling extended to north–eastern Europe and the Balkans with particular stress on the search for the presence of clade E2 and identification of contact zones between Asian and the European clades. Outside the Italy clade E2 has been found only in Istria (Pupcina Cave) on archaeological samples dating to 9000 B.C. As we are not aware of the single contemporary study related to the analysis of mtDNA sequence variation in wild boars sampled in Croatia it is not known if E2 clade is present in Istria and/or other parts of south Croatia.

The present study aims to analyse the nucleotide diversity of CR–mtDNA of wild boars hunted in Istria and Cres Island in order to search for the presence of clade E2 haplotypes outside the Italian peninsula and Sardinia. Our study was based on the archaeological evidence that E2 haplotypes have been documented in Istria (Larson et al., 2007).

Material and methods

A total of 33 blood samples of wild boars from four hunting areas situated in Istria and Cres Island, more precisely from Plominska gora (9), Oprtalj (8), Grožnjan (6) and Tramontana (10) were collected. The DNA was extracted using Sigma blood kit (Sigma–Aldrich, Germany) according to manufacturer's recommendations. Sequencing was done following the procedure described in Montiel–Sosa et al. (2000). Thus, a 497–bp fragment of the pig CR–mtDNA (between nucleotides 15626 – 16123) was amplified using the forward (5'–AACCCTATGTACGTC GTGCAT–3') and reverse (5'–ACCATTGACTGAATAGCACCT–3'), (Invitrogen) primers. The polymerase chain reaction (PCR) protocol was performed in a 20 µl reaction mix containing approximately 50 ng of total DNA, 0.2 µM of each forward and reverse primer and Master Mix (Qiagen). The PCR was carried out in a iCycler (Biorad, USA) thermocycler, and consisted of: an initial denaturation step at 95°C for 9 min followed by 34 cycles at 94°C, for 1 min, annealing at 60°C for 2 min, and elongation at 72°C for 3 min with a final elongation step of 30 min at 72°C. PCR products were purified using ExoSAPIT (USB, Cleveland, OH) following the manufacturer's recommendations. DNA sequencing was performed from the PCR product on an ABI 3130 DNA automated sequencer (Applied Biosystems, USA) using the ABI Prism Big Dye Terminator 3.1 Sequencing Kit (Applied Biosystems, USA). For a more accurate comparison, total 227 sequences were also retrieved from the GeneBank for this study. This was also the reference sequence reported in the GenBank under accession number AJ002189 (Ursing and Arnason, 1998). Obtained mtDNA sequences were aligned using the program CLUSTAL as implemented in the software MEGA 4.1 Beta 3 (Tamura et al., 2007; available at http://www.megasoftware.net/mega41.html). All performed analyses were based on the 428 bp truncated fragment. DnaSP v5.10 (Librado and Rozas, 2009; available at http://www.ub.edu/dnasp/) was used to determine unique haplotypes. The construction of the Neighbour–joining tree was done using Kimura two–parameter model with complete deletion option (Kimura, 1980) by means of MEGA 4.1 Beta 3 software (results not presented).

Results and discussion

The analysis was performed on the 428–bp long sequence fragment in the CR–mtDNA. On a data set of 260 wild boar sequences, 33 typed in this study and 227 retrieved from the GeneBank (Larson et al., 2005; Scandura et al., 2008; Lattuada et al., 2009; Alves et al., 2010), we found 21 different haplotypes. Detailed characterisation of all 21 haplotypes observed is presented in Table 1. Wild boars collected in this study were assigned to two (H2 and H6) haplotypes classified in clade E1, see Table 1. Two wild boars, from Oprtalj and Grožnjan, were assigned in the haplotype H2 while the other 31 samples were assigned in the haplotype H6. On the analyzed fragment, observed haplotypes were two (T→C at 15714 and 15758) and one (T→C at 15758) transversions remote from the H1 reference sequence reported in the GenBank under accession number AJ002189 (Ursing and Arnason, 1998). The rare haplotype (H2) conforms to the haplotype H2 from Lattuade et al. (2009) identified in four Italian wild boar populations (Arezzo, Bergamo, Parma and Pisa), south–west European populations (France, Portugal and Spain) as well as in a large number of domestic pig populations (Berkshire, Duroc, Iberian Black pig, Landrace and Mangalica). On the sequence restriction made in this analysis H2 conforms to haplotypes S022, S023 and S029 found in Alves et al. (2010) on the 411–bp long sequence fragment. Dominant haplotype H6 was also identified in Portugal (3/36), Spain (10/93) and very rarely in Italy (1/89), see Table 2. Haplotypes S021 and S083, found in Alves et al. (2010) on the 411–bp long sequence fragment, conform to this study H6 haplotype. While only one sample from Italy was assigned to haplotype H6, there were nine samples from Goriza, neighboring north–east region of Italy, that were assigned to the haplotype H15. As haplotype H15 is just one or two mutations remote from the H2 and H6 haplotype, the results obtained pointed to the possible closeness between wild boar populations from Goriza, Istria and Cres Island. Based on 14 microsatellite information, Šprem (2009) has shown that wild boars from Istria and Cres Island form one separated population, also partially present across Velebit and Gorski Kotar. For fur-

Table 1. Polymorphic nucleotide positions within 428–bp fragment of the mitochondrial DNA control region found in 260 wild boars sampled in different European countries. Nucleotide positions are numbered according to the reference sequence GenBank AJ002189 (Ursing and Arnason, 1998)

Haplotypes	411-bp fragment (Alves et al. 2010)	15674	15675	15711	15714	15723	15729	15737	15741	15758	15822	15825	15840	15878	15887	15936	15995	16010	16032	16045
H1 (AJ002189)	S035, S119	T	T	T	T	A	A	A	C	T	A	C	T	A	C	A	T	A	G	T
H2	S022, S023, S029	.	.	.	C	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.
H3	S085	.	.	.	.	.	.	.	.	C	.	T	.	.	.	.	.	.	.	.
H4	S085	.	.	.	.	.	.	.	.	C	.	T	.	G	.	.	.	.	.	.
H5	S021	.	.	.	C	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.
H6	S021, S083	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H7	S115	.	.	.	C	.	.	.	.	C	G	.	.	.	.	.	.	.	.	.
H8	S056	.	.	.	.	.	.	.	.	C	G	.	.	.	.	.	.	.	.	.
H9	S029	.	.	.	C	.	.	.	.	C	.	.	.	.	.	G	.	.	.	.
H10	S117	C	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H11	S116	.	.	.	C	G	.	.	.	C	G	.	.	.	.	.	.	.	.	.
H12	S054	.	.	.	C	G	.	.	.	C	.	.	.	.	.	G	.	.	.	.
H13	S117	.	.	.	C	.	.	.	T	C	.	.	.	.	.	.	.	.	.	.
H14		.	.	.	.	.	.	G	.	C	.	T	.	.	.	.	.	.	.	.
H15		.	.	.	C	.	.	G	.	C	.	.	.	.	.	.	.	.	.	.
H16		.	.	.	C	.	.	.	.	C	.	.	.	G	.	.	.	.	.	.
H17		.	C	.	C	.	G	.	T	.	.	T	C	.	T	.	.	G	.	.
H18	S011	.	.	C	.	.	G	.	.	.	.	.	.	G	.	.	C	G	.	G
H19		.	C	.	C	.	G	.	T	.	.	T	C	.	T	.	.	G	.	G
H20	S069, S012	.	.	C	.	.	G	.	.	C	.	.	.	G	.	.	C	G	.	G
H21	S012	.	.	C	.	.	G	.	.	C	.	.	.	G	.	.	C	G	T	G

Table 2. Distribution of haplotype frequencies of the 428–bp fragment of the mitochondrial DNA control region in 260 wild boars sampled in different European countries

Haplotype	Portugal	Spain	Austria	France	Italy/R	Italy/G	Croatia	Total
H1			3		1			4
H2	7	14		2	35		2	60
H3		3		2	5			10
H4				2				2
H5	1	18						19
H6	3	10			1		31	45
H7	3	15						18
H8	8							8
H9	14							14
H10		1						1
H11		11						11
H12		16						16
H13		5						5
H14					6			6
H15						9		9
H16					7			7
H17					13			13
H18					6			6
H19					2			2
H20					3			3
H21					1			1

Italy/R refers to all regions in Italy except Gorizia;
Italy/G refers to the north-eastern Italian region, Gorizia

ther research, we propose a hypothesis that wild boars populations placed in Istria, Cres Island, Slovenia and Goriza form one population. Still, this hypothesis has to be tested with analyses based on nuclear markers such as microsatellites or/and SNPs.

Unfortunately, on the sequence analyzed, we were not able to make distinction between S022, S023 and S029 (here H2) nor between S021 and S083 (here H6) that would provide more informative results. Thus, we plan to extend our analyses to the large number of wild boars distributed all over Croatia and neighboring countries as well as to a larger mtDNA sequence fragment.

Conclusions

In analyses performed on the 33 wild boars hunted in Istria and Cres Island, we have observed two haplotypes (H2 and H6), both, identified as haplotypes of E1 clade that is known to be present in European wild and domestic pigs. While we cannot exclude the possibility that clade E2 occurs at a low frequency in Croatia, the results obtained here further support the indigenous status (Italy) of the clade E2. Furthermore, of the results obtained indicate drastic changes in the maternal origin of the wild boars in Istria and Cres Island during last 11000 years. Obtained results also pointed to the closeness of the maternal origin between wild boars hunted in north–eastern Italy (Gorizia) and those hunted in Istria and Cres Island, leaving possibility they might form one population.

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