

Mitochondrial variability of Small Međimurje dog

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Summary

There are six native dog breeds in Croatia recognized by the Federation Cynologique International (FCI) and one national dog breed Small Međimurje dog (MEDI) still unrecognized by the FCI. To promote breed we have analysed mitochondrial DNA control region (CR-mtDNA) sequence (551-bp) in 35 Small Međimurje dogs sampled in Međimurje County. After comparison with 33 worldwide distributed dog breeds (N=115 samples), three main canine CR-mtDNA haplogroups (A, B and C) were observed in Small Međimurje dogs. Median-joining tree showed that MEDI forms six haplotypes presented in haplogroup C (H3 is the most frequent in MEDI population), haplogroup A (haplotypes H2, H5 and H7) and in haplogroup B (haplotypes H4 and H6). The results presented in this study correspond to other mtDNA studies of native dog breeds. For the better genetic description of MEDI and for the optimal future breeding management, further analyses of nuclear genome are recommended.

Key words

Small Međimurje dog, mitochondrial DNA, haplotype diversity

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Introduction

Fédération Cynologique Internationale (FCI) recognizes six Croatian native dog breeds: Dalmatian (DALM), Croatian Shepherd (CROS), Istrian Short-haired Hound (ISTKG), Istrian Wire-haired Hound (ISTOG), Posavatz Hound (POSG) and Tornjak (TOR). The Croatian Kennel Association officially recognized Small Međimurje dog (MEDI) as a Croatian native breed in 2010, but it is still not internationally FCI recognized. MEDI has been bred for over a hundred years in Međimurje, a region of northern part of Croatia placed between rivers Mura and Drava. MEDI can usually be found in rural households, where it serves as a guard dog and/or destroys harmful rodents. It belongs to a lupoid type of dogs and has elevated ears. It is a small, very closely-structured dog, rectangular in shape with short legs. The coat is flat, hard, short or medium long. MEDI comes in tricolour or bicolour with different patches (Fig 1. b, c). There is no information about the population size of MEDI and there is no single genetic analysis of the origin and/or diversity of MEDI (Hrvatski kinološki savez, <https://web.hks.hr/mali-medimurski-pas/>) to our knowledge.

The genetic structure of the domestic dog has been investigated using microsatellites, SNP chips but most frequently mtDNA (Vilà et al., 1999). The length of complete referent dog mitogenome is 16,727 bp (Kim et al., 1998). Many analyses of canine mtDNA diversity were done on control region (D-loop) (Scharnhorst and

Kanthaswamy, 2011) due to its high variability. Canine control region (CR) of mtDNA is approximately 1270 bp long (Kim et al., 1998).

Webb and Allard (2009) were the first authors to analyse the whole CR of canine mtDNA. Later Imes et al. (2012) determined 71 single nucleotide polymorphisms (SNP) in CR of canine mtDNA that divides it into six main CR haplogroups (A, B, C, D, E and F), named after Savolainen et al. (2002). Haplogroups A, B and C contain 95% of all dog haplotypes and are presented worldwide with exception of Americas for haplogroups B and C (Pires et al., 2006; Verscheure et al., 2013). According to Pang et al. (2009) haplogroups D, E and F are found only regionally, possibly from crossbreeding with wolf. Haplogroup E is found in Southeast Asia, Korea and Japan and it is therefore possible that it originated from the early processes of domestication of dogs together with clades A, B and C, but did not spread out of East Asia like the other haplogroups. Haplogroup F is found in dogs sampled in Japan. Haplogroup D seems to have two separate origins from wolf, since one subhaplogroup of sequences is found only in North Scandinavian dogs and the other subhaplogroup in South-west Asia, North Africa and Iberia.

The aim of the present study was to characterize maternal genetic diversity of MEDI in Croatia, to determine their haplogroups and to compare it with other dog breeds.

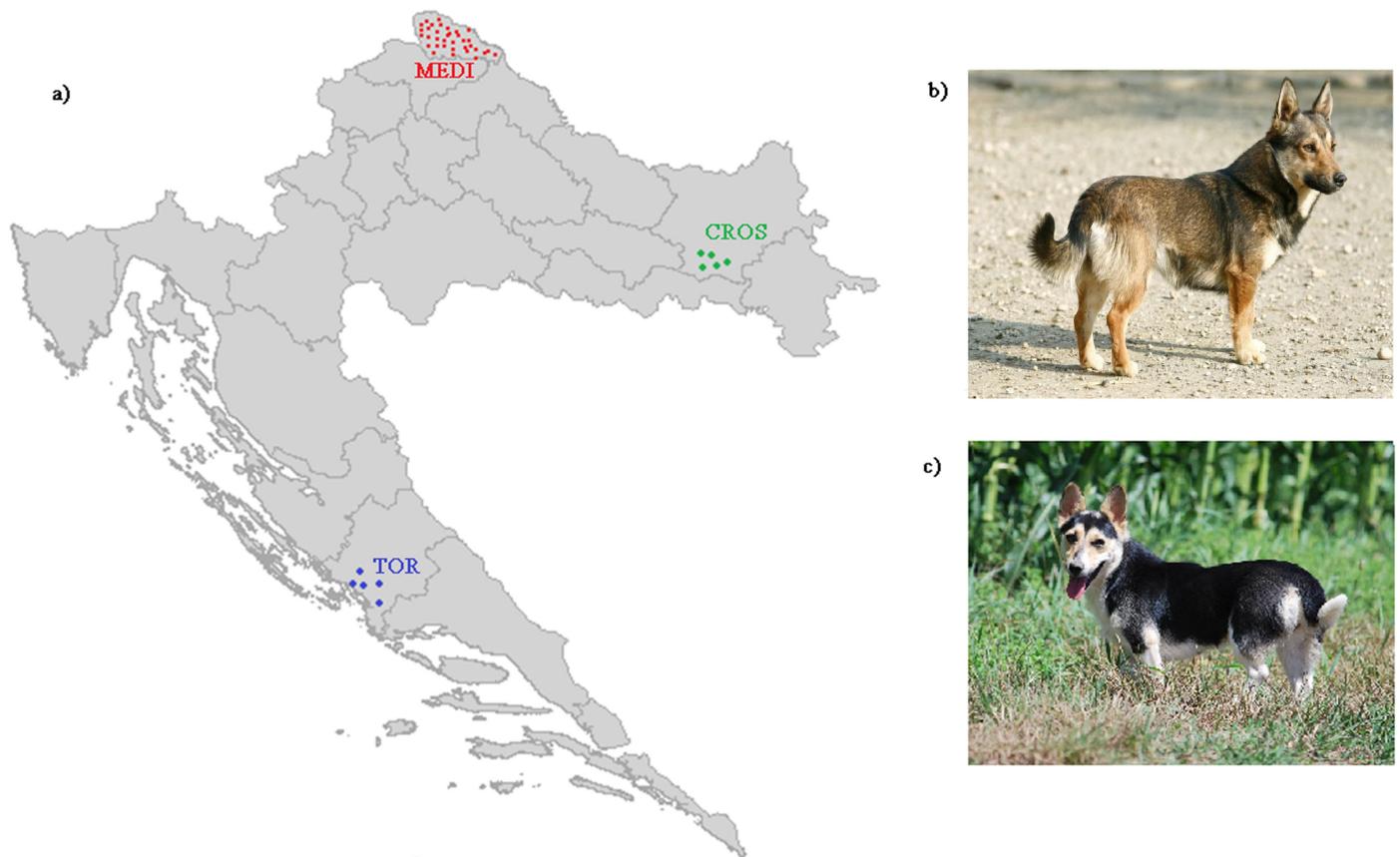


Figure 1. a) Sampling location: Small Međimurje dog (MEDI), Croatian Shepherd dog (CROS) and Tornjak (TOR); b) Small Međimurje dog male (source: <https://mali-medjimurski-pas-medji.weebly.com/photogallery.html>); c) Small Međimurje dog female (source: <https://mali-medjimurski-pas-medji.weebly.com/photogallery.html>).

Materials and methods

Sampling

A total of 49 buccal swab samples (39 MEDI, 5 CROS and 5 TOR) were collected at dog shows or directly from breeders and owners (Fig. 1a). Identification of purebred animals was based on specific morphological criteria and/or pedigree.

DNA and PCR analysis

DNA was isolated using DNeasy Blood & Tissue Reagent Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The quantity of DNA was evaluated using NanoVue (GE Healthcare Life Sciences, USA). The 680 bp mtDNA control region was amplified using forward H15422: (5'-CTCTTGCTCCACCATCAGC-3') and reverse L16106: (5'-AAACTATATGTCCTGAAACC-3') primers according to PCR conditions described in Boyko et al (2009). PCR products were purified using Wizard SV Gel and PCR Clean-Up Kit (Promega, USA).

Sequence analysis

Sequencing reactions were carried out on an ABI 3130 DNA automated sequencer (Applied Biosystems, USA), using BigDye Terminator 3.1 Sequencing Kit (Applied Biosystems, USA) and with both primers. Electropherograms were visualized, edited, assembled, and aligned with MEGA 7 (Kumar et al., 2016). After check-up, any reads with ambiguous bases were edited and/or removed and our final dataset finished up with 35 MEDI, 5 CROS and 5 TOR which were deposited into the GenBank under accession numbers MK852741 - MK852785. Since there is no record of breeds participating in the establishment of MEDI, for comparison, we chose dog breeds that are geographically close, physically similar and of similar utilisation to MEDI from publically available sequences (115 sequences from GenBank). For the purpose of this study dogs were grouped based on their utilisation (Table 1). Referent sequence of complete dog mtDNA sequence used in this study is stored under GeneBank accession number: NC_002008 (Kim et al., 1998).

Table 1. Accession numbers, breeds, utilisation groups and references of the mtDNA CR sequences included in the alignment in the present study.

Accession Number	Breed	Utilisation group	Reference
MK852741-MK852745	Croatian Shepherd dog	Sheepdog	this study
MK852746-MK852780	Small Medimurje dog	Sheepdog	this study
MK852781-MK852785	Tornjak	Livestock guardian dog	this study
HQ845282; HQ997437	Cocker Spaniel	Hunting dog	Bekaert et al. (2012)
HQ845273	German Bracke	Hunting dog	Bekaert et al. (2012)
HQ997590	Labrador	Hunting dog	Bekaert et al. (2012)
HQ997538; HQ997539	Yorkshire Terrier	Terrier	Bekaert et al. (2012)
EU223392-EU223394	Airedale Terrier	Terrier	Webb and Allard (2009)
EU223397-EU223399; EU223401-EU223404	Australian Shepherd	Sheepdog	Webb and Allard (2009)
EU223395	Australian Terrier	Terrier	Webb and Allard (2009)
EU223455-EU223462	Basset Hound	Hunting dog	Webb and Allard (2009)
EU223416; EU223418; EU223421-EU223423	Beagle	Hunting dog	Webb and Allard (2009)
EU223442-EU223444; EU223446-EU223448	Border Collie	Sheepdog	Webb and Allard (2009)
EU223504; EU223505	Cavalier King Charles Spaniel	Hunting dog	Webb and Allard (2009)
EU223509	Collie	Sheepdog	Webb and Allard (2009)
EU223476; EU223514; EU223515; EU223517; EU223662; EU223797	Corgi	Sheepdog	Webb and Allard (2009)
EU223520-EU223522; EU223524	Cocker Spaniel	Hunting dog	Webb and Allard (2009)
EU223535	Dachshund	Hunting dog	Webb and Allard (2009)
EU223542-EU223544	Dalmatian	Hunting dog	Webb and Allard (2009)
EU223558	Fox terrier	Terrier	Webb and Allard (2009)

Accession Number	Breed	Utilisation group	Reference
EU223592-EU223595; EU223598; EU223599	Jack Russell Terrier	Terrier	Webb and Allard (2009)
EU223481-EU223486	Labrador	Hunting dog	Webb and Allard (2009)
EU223650; EU223651	Manchester Terrier	Terrier	Webb and Allard (2009)
EU223681; EU223683; EU223684; EU223686; EU223688; EU223690	Poodle	Hunting dog	Webb and Allard (2009)
EU223711	Rat Terrier	Terrier	Webb and Allard (2009)
EU223775	Toy Fox Terrier	Terrier	Webb and Allard (2009)
EU223789; EU223792-EU223795	West Highland White Terrier	Terrier	Webb and Allard (2009)
EU223808-EU223811	Yorkshire Terrier	Terrier	Webb and Allard (2009)
HQ997586	Australian Shepherd	Sheepdog	Bekaert et al. (unpublished)
HQ997521	Belgian Groenendaal	Sheepdog	Bekaert et al. (unpublished)
HQ997555	Belgian Malinois	Sheepdog	Bekaert et al. (unpublished)
HQ997531	Beagle	Hunting dog	Bekaert et al. (unpublished)
HQ997467; HQ997516; HQ997571	Cairn Terrier	Terrier	Bekaert et al. (unpublished)
HQ997481; HQ997519; HQ997526	Cavalier King Charles Spaniel	Hunting dog	Bekaert et al. (unpublished)
HQ997503	Collie	Sheepdog	Bekaert et al. (unpublished)
HQ997552	Cocker Spaniel	Hunting dog	Bekaert et al. (unpublished)
HQ997479	Dalmatian	Hunting dog	Bekaert et al. (unpublished)
HQ997507	German Shepherd	Sheepdog	Bekaert et al. (unpublished)
HQ997443; HQ997448; HQ997451; HQ997471; HQ997500; HQ997502; HQ997554; HQ997557; HQ997562	Golden Retriever	Hunting dog	Bekaert et al. (unpublished)
HQ997497; HQ997547; HQ997550	Labrador	Hunting dog	Bekaert et al. (unpublished)
HQ997458; HQ997515; HQ997525	Yorkshire Terrier	Terrier	Bekaert et al. (unpublished)

Phylogenetic analysis

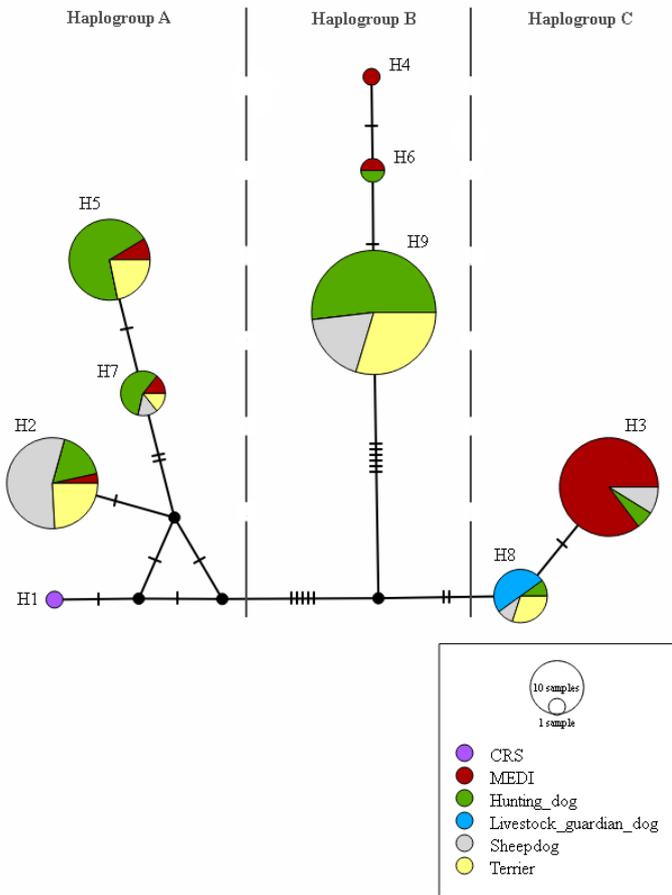
Haplogroup classification was done using MitoToolPy program (Peng et al., 2015). Haplotypes were calculated using DnaSP 5.10 (Librado and Rozas, 2009) and median-joining network (Bandelt et al., 1999) was constructed by PopART (Leigh and Bryant, 2015). The principal component analysis (PCA) was carried out on the CR of canine mtDNA sequences to determine breed relationships. PCA was performed using easyGgplot2 package for R Software (Kassambara, 2014).

Results and discussion

In the analysis based on 160 sequences of CR-mtDNA 551 bp long, we identified eight different haplotypes. Phylogenetic analysis is presented with the median-joining tree in Fig. 2. Canine referent mtDNA sequence represents haplotype H1 in haplogroup A. The haplogroup A is presented with four haplotypes (H1, H2, H5 and H7) while the haplogroup B is presented by three haplotypes (H4,

H6 and H9) and haplogroup C, by two haplotypes (H3 and H8). MEDI is presented in haplogroup A (haplotypes H2, H5 and H7), haplogroup B (haplotypes H4 and H6) and it forms haplotype H3 in haplogroup C and that is the most frequent haplotype for MEDI.

The three haplogroups (A, B and C) were clearly separated, while MEDI appeared in all three of them and every haplogroup was composed of all four utilisation dog breed groups used in this study (Hunting, Livestock guardian, Sheepdog and Terrier group of dogs). This is consistent with results presented in various dog population studies. In general, dogs are not grouped in their respective breed according to their mtDNA haplogroups, because of haplotype sharing (Gundry et al., 2007; Parra et al., 2008; Desmyter and Comblez, 2009; Kropatsch et al., 2011; Desmyter and Gijsbers, 2012; Suárez et al., 2013). Haplotype frequencies can differ between specific breeds and the entire dog mtDNA gene pool, as well as between breeds (Gundry et al., 2007; Parra et al., 2008; Webb and Allard, 2009; Kropatsch et al., 2011; Desmyter



and Gijsbers, 2012; Suárez et al., 2013). Haplotype H9 is most frequent and it appears in Hunting (28/54), Sheepdogs (10/54) and Terrier (16/54) group of dogs. MEDI is present in haplotypes H2, H3, H4, H5, H6 and H7 where H4 is unique haplotype and others are shared. MEDI (29/34) forms haplotype H3, but there are also samples from Sheepdog (3/34) and Hunting (2/34) dog group in H3. Also, one MEDI shares haplotype H6 with Hunting (1/2) dog group, haplotype H7 with Shepherd (1/7), Terrier (1/7) and Hunting (4/7) dog groups, and haplotype H2 with Shepherd (16/29), Terrier (7/29) and Hunting (5/29) dog group. Two MEDIs are also placed in haplotype H5 with Hunting (16/23) and Terrier (5/23) group of dogs. Positions of polymorphisms of the 551-bp fragment of mitochondrial DNA control region of dog breeds from our dataset are shown in the Table 2.

In principal component analysis MEDI was in the cloud of Hunting, Terrier and Sheepdog group of dogs (Fig. 3). It is remote to other Croatian native dog breeds (Croatian Shepherd dog, Dalmatian, and Tornjak) that form the other cloud on the right part of the graph. Other breeds close to MEDI are Cavalier King Charles Spaniel, Jack Russel Terrier, Australian Shepherd, Labrador Retriever, Border Collie, Cocker Spaniel, Golden Retriever, Beagle, Yorkshire Terrier, and West Highland White Terrier.

Figure 2. The median-joining (MJ) network for the 551-bp data set showing phylogenetic positions of haplotypes found in Small Medimurje dog comparing to haplotypes found in other dog breeds. The names of the haplotypes are displayed on the graph containing haplotype letter and number. Coloured circles represent: referent canine mtDNA (CRS) as dark blue, MEDI as red, Hunting dog breed group as green (representing breeds: Basset Hound, Beagle, Cavalier King Charles Spaniel, Cocker Spaniel, Dachshund, Dalmatian, German Bracke, Golden Retriever, Labrador Retriever and Poodle), Livestock guardian dog breed group as light blue (representing Tornjak), Sheepdog breed group as grey (representing Corgi, Croatian Shepher dog, Belgian Groenendaal, Belgian Malinois, Border Collie and German Shepherd dog) and Terrier breed group as yellow circle (representing Airdale Terrier, Australian Terrier, Cairn Terrier, Fox Terrier, Jack Russel Terrier, Manchester Terrier, Rat Terrier, Toy Fox Terrier, West Highland White Terrier and Yorkshire Terrier). Numbers of mutations separating nodes are given near branches in parentheses.

Table 2. Positions of polymorphisms of the 551-bp fragment of mitochondrial DNA control region in 160 samples of world known dog breeds. Nucleotide positions are numbered according to the GenBank dog referent sequence, RRS: NC_002008 (Kim et al., 1998) within haplotype H1. MEDI is present in haplotypes H2, H3, H4, H5, H6 and H7 and they are marked as bold.

	15526	15595	15611	15612	15620	15627	15632	15639	15643	15650	15652	15710	15800	15814	15815	15912	15955	16003	16025	16042
H1	C	C	T	T	T	A	C	T	A	T	G	C	T	C	T	C	C	A	T	T
H2	A	T	C	.
H3	T	.	C	G	.	C	.	T	C	T	.	T	T	G	.	.
H4	T	T	.	C	.	.	T	G	G	.	A	.	C	T	C	T	T	.	.	A
H5	C	G	.	A	T	.	.	T	.	.	.
H6	T	T	.	C	.	.	T	G	G	.	A	.	C	T	C	T	T	.	.	.
H7	C	G	.	A	T
H8	T	.	C	G	.	C	.	.	C	T	.	T	T	G	.	.
H9	T	T	.	C	.	.	T	G	G	.	A	.	C	T	C	T	T	G	.	.

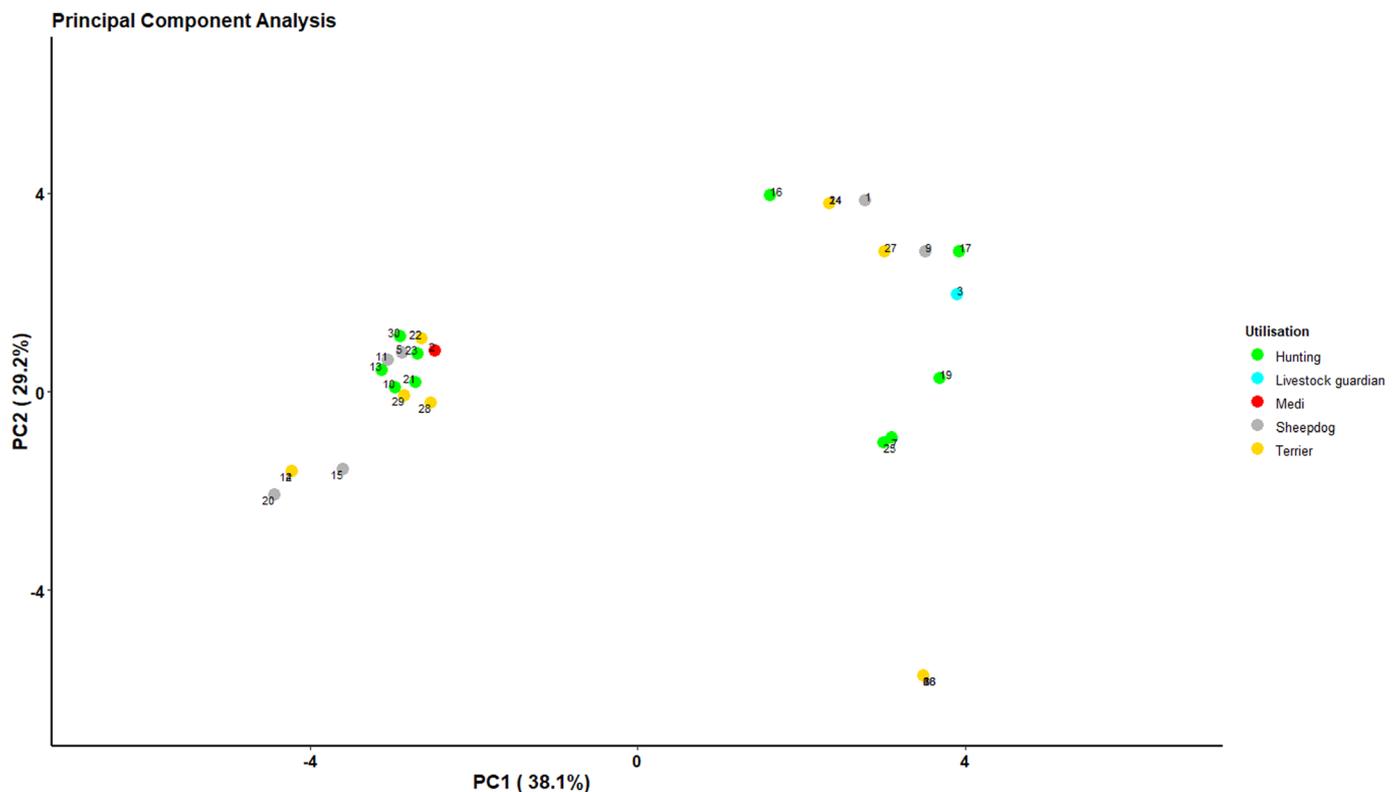


Figure 3. Principal component analysis (PCA) determines breed relationships between Small Međimurje dog and groups of dogs based on their utilisation. Hunting group is represented as green dots, Livestock guardian group as cyan dot, MEDI as red dot, Sheepdog group as grey dots and Terrier group as yellow dots. Numbers represent breeds (1 – Croatian Shepherd dog, 2 – Small Medimurje dog, 3 – Tornjak, 4 – Airdale Terrier, 5 – Australian Shepherd dog, 6 – Australian Terrier, 7 – Basset Hound, 8 – Belgian Groenendal, 9 – Belgian Malinois, 10 – Beagle, 11 – Border Collie, 12 – Cairn Terrier, 13 – Cocker Spaniel, 14 – Collie, 15 – Corgi, 16 – Dachshund, 17 – Dalmatian, 18 – Fox Terrier, 19 – German Bracke, 20 – German Shepherd, 21 – Golden Retriever, 22 – Jack Russel Terrier, 23 – Labrador Retriever, 24 – Manchester Terrier, 25 – Poodle, 26 – Rat Terrier, 27 – Toy Fox Terrier, 28 – West Highland White Terrier, 29 –Yorkshire Terrier, 30 – Cavalier King Charles Spaniel).

Conclusion

This study is the first genetic analysis of phylogenetic relationship of Small Međimurje dogs in Croatia. Small Međimurje dog breed is a small population of Croatian native dogs still fighting to become internationally FCI recognized. Therefore it needs a detailed genetic analysis and clearly defined breeding objectives. According to our analysis of CR of mtDNA, Small Međimurje dog is classified into three different haplogroups, but mostly in C. According to PCA it falls into cloud of Hunting, Terrier and Sheepdog group of dogs which corresponds to their selection goal and utilisation history where Small Međimurje dog usually served as a guard dog and/or destroys harmful rodents in rural households. For the better genetic description of MEDI and for the optimal future breeding management, further analyses of nuclear genome are recommended.

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