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1 **Mitochondrial variability of Small Međimurje dog**

2

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13

14 **Abstract**

15

16 There are six native dog breeds in Croatia recognized by the Federation cynologique
17 international (FCI) and one national dog breed Small Međimurje dog (MEDI) still unrecognized
18 by the FCI. To promote breed we have analysed mitochondrial DNA control region (CR-
19 mtDNA) sequence (551-bp) in 35 Small Međimurje dogs sampled in Međimurje County. After
20 comparison with 33 worldwide distributed dog breeds (N=115 samples), three main canine CR-
21 mtDNA haplogroups (A, B and C) were observed in Small Međimurje dogs. Median-joining
22 tree showed that MEDI forms six haplotypes presented in haplogroup C (H3 is the most
23 frequent in MEDI population), haplogroup A (haplotypes H2, H5 and H7) and in haplogroup B
24 (haplotypes H4 and H6). The results presented in this study correspond to other mtDNA studies

25 of native dog breeds. For the better genetic description of MEDI and for the optimal future
26 breeding management, further analysis of nuclear genome are recommended.

27

28 **Keywords:** Small Međimurje dog, mitochondrial DNA, haplotype diversity

29

30 **Introduction**

31

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

45 The genetic structure of the domestic dog has been investigated using microsatellites, SNP chips
46 but most frequently mtDNA (Vilà et al., 1999). The length of complete referent dog
47 mitogenome is 16,727 bp (Kim et al., 1998). Many analyses of canine mtDNA diversity were
48 done on control region (D-loop) (Scharnhorst and Kanthaswamy, 2011) due to its high

49 variability. Canine control region (CR) of mtDNA is approximately 1,270 bp long (Kim et al.,
50 1998).
51 Webb and Allard (2009) were the first authors to analyse the whole CR of canine mtDNA. Later
52 Imes et al (2012) determined 71 single nucleotide polymorphisms (SNP) in CR of canine
53 mtDNA that divides it into six main CR haplogroups (A, B, C, D, E and F), named after
54 Savolainen et al (2002). Haplogroups A, B and C contain 95% of all dog haplotypes and are
55 presented worldwide with exception of Americas for haplogroups B and C (Pires et al., 2006,
56 Verscheure et al., 2013). According to Pang et al (2009) haplogroups D, E and F are found only
57 regionally, possibly from crossbreeding with wolf. Haplogroup E is found in Southeast Asia,
58 Korea and Japan and it is therefore possible that it originated from the early processes of
59 domestication of dogs together with clades A, B and C, but did not spread out of East Asia like
60 the other haplogroups. Haplogroup F is found in dogs sampled in Japan. Haplogroup D seems
61 to have two separate origins from wolf, since one subhaplogroup of sequences is found only in
62 North Scandinavian dogs and the other subhaplogroup in South-west Asia, North Africa and
63 Iberia.

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

66

67 **Materials and methods**

68

69 **Sampling**

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

73 **DNA and PCR analysis**

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

81

82 **Sequence analysis**

83 Sequencing reactions were carried out on an ABI 3130 DNA automated sequencer (Applied
84 Biosystems, USA), using BigDye Terminator 3.1 Sequencing Kit (Applied Biosystems, USA)
85 and with both primers. Electropherograms were visualized, edited, assembled, and aligned with
86 MEGA 7 (Kumar et al., 2016). After check-up, any reads with ambiguous bases were edited
87 and/or removed and our final dataset finished up with 35 MEDI, 5 CROS and 5 TOR which
88 were deposited into the GenBank under accession numbers MK852741 - MK852785. Since
89 there is no record of breeds participating in the establishment of MEDI, for comparison, we
90 chose dog breeds that are geographically close, physically similar and of similar utilisation to
91 MEDI from publically available sequences (115 sequences from GenBank). For the purpose of
92 this study dogs were grouped based on their utilisation (Table S1). Referent sequence of
93 complete dog mtDNA sequence used in this study is stored under GeneBank accession number:
94 NC_002008 (Kim et al., 1998).

95

96

97 **Phylogenetic analysis**

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

104

105 **Results and discussion**

106

107 In the analysis based on 160 sequences of CR-mtDNA 551 bp long, we identified eight different
108 haplotypes. Phylogenetic analysis is presented with the median-joining tree in Fig. 2. Canine
109 referent mtDNA sequence represents haplotype H1 in haplogroup A. The haplogroup A is
110 presented with four haplotypes (H1, H2, H5 and H7) while the haplogroup B is presented by
111 three haplotypes (H4, H6 and H9) and haplogroup C, by two haplotypes (H3 and H8). MEDI
112 is presented in haplogroup A (haplotypes H2, H5 and H7) and haplogroup B (haplotypes H4
113 and H6) and it forms haplotype H3 in haplogroup C and that is the most frequent haplotype for
114 MEDI.

115 The three haplogroups (A, B and C) were clearly separated, while MEDI appeared in all three
116 of them and every haplogroup was composed of all four utilisation dog breed groups used in
117 this study (Hunting, Livestock guardian, Sheepdog and Terrier group of dogs). This is
118 consistent with results presented in various dog population studies. In general, dogs are not
119 grouped in their respective breed according to their mtDNA haplogroups, because of haplotype
120 sharing (Gundry et al., 2007; Parra et al., 2008; Desmyter and Comblez, 2009; Kropatsch et

121 al., 2011; Desmyter and Gijbers, 2012; Suárez et al., 2013). Haplotype frequencies can differ
122 between specific breeds and the entire dog mtDNA gene pool, as well as between breeds
123 (Gundry et al., 2007; Parra et al., 2008; Webb and Allard, 2009; Kropatsch et al., 2011;
124 Desmyter and Gijbers, 2012; Suárez et al., 2013). Haplotype H9 is most frequent and it appears
125 in Hunting (28/54), Sheepdogs (10/54) and Terrier (16/54) group of dogs. MEDI is present in
126 haplotypes H2, H3, H4, H5, H6 and H7 where H4 is unique haplotype and others are shared.
127 MEDI (29/34) forms haplotype H3, but there are also samples from Sheepdog (3/34) and
128 Hunting (2/34) dog group in H3. Also, one MEDI shares haplotype H6 with Hunting (1/2) dog
129 group, haplotype H7 with Shepherd (1/7), Terrier (1/7) and Hunting (4/7) dog groups, and
130 haplotype H2 with Shepherd (16/29), Terrier (7/29) and Hunting (5/29) dog group. Two MEDIs
131 are also placed in haplotype H5 with Hunting (16/23) and Terrier (5/23) group of dogs.
132 Positions of polymorphisms of the 551-bp fragment of mitochondrial DNA control region of
133 dog breeds from our dataset are shown in the Table 1.

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

140

141 **Conclusion**

142

143 This study is the first genetic analysis of phylogenetic relationship of Small Međimurje dogs in
144 Croatia. Small Međimurje dog breed is a small population of Croatian native dogs still fighting

145 to become internationally FCI recognized. Therefore it needs a detailed genetic analysis and
146 clearly defined breeding objectives. According to our analysis of CR of mtDNA, Small
147 Međimurje dog is classified into three different haplogroups, but mostly in C. According to
148 PCA it falls into cloud of Hunting, Terrier and Sheepdog group of dogs which corresponds to
149 their selection goal and utilisation history where Small Međimurje dog usually served as a guard
150 dog and/or destroys harmful rodents in rural households. For the better genetic description of
151 MEDI and for the optimal future breeding management, further analysis of nuclear genome are
152 recommended.

153

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155

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157 samples and information about this beautiful breed. In particular, we thank Croatian Kennel
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159 selfless assistance and expert guidance in selecting samples and reviewers for valuable
160 comments.

161

162 **References**

163

164 Bandelt H. J., Forster P., Rohlf A. (1999). Median-joining networks for inferring intraspecific
165 phylogenies. *Mol Biol Evol.* 16: 37-48. doi: 10.1093/oxfordjournals.molbev.a026036

166 Boyko A. R., Boyko R. H., Boyko C. M., Parker H. G., Castelhano M., Corey L., Degenhardt
167 J. D., Auton A., Hedimbi M., Kityo R., Ostrander E. A., Schoenebeck J., Todhunter R. J., Jones
168 P., Bustamante C. D. (2009). Complex population structure in African village dogs and its

169 implications for inferring dog domestication history. *Proceedings of the National Academy of*
170 *Sciences* 106: 13903-13908. doi: 10.1073/pnas.0902129106

171 Desmyter S., Comblez S. (2009). Belgian dog mitochondrial DNA database for forensics.
172 *Forensic Sci Int: Genetics Suppl Ser 2*: 286-287.

173 Desmyter S., Gijsbers L. (2012). Belgian canine population and purebred study for forensics
174 by improved mitochondrial DNA sequencing. *Forensic Sci Int: Genetics* 6: 113-120. doi:
175 10.1016/j.fsigen.2011.03.011

176 Gundry R. L., Allard M. W., Moretti T. R., Honeycutt R. L., Wilson M. R., Monson K. L.,
177 Foran D.R. (2007). Mitochondrial DNA Analysis of the Domestic Dog: Control Region
178 Variation Within and Among Breeds. *Forensic Sci Int: Genetics Suppl Ser 52*: 562-572.

179 Hassell R., Heath P., Musgrave-Brown E., Ballard D., Harrison C., Thacker C., Catchpole B.,
180 Syndercombe-Court D. (2008). Mitochondrial DNA analysis of domestic dogs in the UK.
181 *Forensic Sci Int: Genetics Suppl Ser 1*: 598–599.

182 Imes D. L., Wictum E. J., Allard M. W., Sacks B. N. (2012). Identification of single nucleotide
183 polymorphisms within the mtDNA genome of the domestic dog to discriminate individuals with
184 common HVI haplotypes. *Forensic Sci Int: Genetics* 6: 630-639.

185 Kassambara A. (2014). *easyGgplot2: Perform and customize easily a plot with ggplot2*. R
186 package version 1.0.0.9000. <http://www.sthda.com>

187 Kim K. S., Lee S. E., Jeong H. W., Ha J. H. (1998). The Complete Nucleotide Sequence of the
188 Domestic Dog (*Canis familiaris*) Mitochondrial Genome. *Mol Phylogenet Evol.* 10: 210-220.
189 doi: 10.1006/mpev.1998.0513

190 Kropatsch R., Streitberger K., Schulte-Middelmann T., Dekomien G., Epplen J. T. (2011). On
191 ancestors of dog breeds with focus on Weimaraner hunting dogs. *J Anim Breed Genet* 128: 64-
192 72. doi: 10.1111/j.1439-0388.2010.00874.x

193 Kumar S., Stecher G., Tamura K. (2016). MEGA7: Molecular Evolutionary Genetics Analysis
194 Version 7.0 for Bigger Datasets. *Mol Biol Evol.* 33: 1870-1874. doi: 10.1093/molbev/msw054
195 Leigh J. W., Bryant D. (2015). popart: full-feature software for haplotype network construction.
196 *Methods in Ecology and Evolution* 6: 1110-1116. doi: 10.1111/2041-210X.12410
197 Librado P., Rozas J. (2009). DnaSP v5: a software for comprehensive analysis of DNA
198 polymorphism data. *Bioinformatics* 25: 1451-1452. Doi: 10.1093/bioinformatics/btp187
199 MALI MEĐIMURSKI PAS. Available at: <https://web.hks.hr/mali-medimurski-pas/Hrvatski>
200 kinološki savez [Accessed 24.04.2019.]
201 Pang J. F., Kluetsch C., Zou X. J., Zhang A. B., Luo L. Y., Angleby H., Ardalan A., Ekström
202 C., Sköllermo A., Lundeberg J., Matsumura S., Leitner T., Zhang Y. P., Savolainen P. (2009).
203 mtDNA Data Indicate a Single Origin for Dogs South of Yangtze River, Less Than 16,300
204 Years Ago, from Numerous Wolves. *Mol Biol Evol.* 26: 2849-2864. doi:
205 10.1093/molbev/msp195
206 Parra D., Méndez S., Cañón J., Dunner S. (2008). Genetic differentiation in pointing dog breeds
207 inferred from microsatellites and mitochondrial DNA sequence. *Anim Genet* 39: 1-7. doi:
208 10.1111/j.1365-2052.2007.01658.x
209 Peng M. S., Fan L., Shi N. N., Ning T., Yao Y. G., Murphy R. W., Wang W. Z., Zhang Y. P.
210 (2015). DomeTree: a canonical toolkit for mitochondrial DNA analyses in domesticated
211 animals. *Mol Ecol Resour* 15: 1238-1242. doi: 10.1111/1755-0998.12386
212 R Core Team R: A Language and Environment for Statistical Computing. 2016. Available at:
213 <https://www.R-project.org/> Available at. [Accessed 25.04.2019.]
214 Savolainen P., Zhang Y. P., Luo J., Lundeberg J., Leitner T. (2002). Genetic Evidence for an
215 East Asian Origin of Domestic Dogs. *Science.* 298: 1610-1613. Doi: 10.1126/science.1073906

216 Scharnhorst G., Kanthaswamy S. (2011). An assessment of scientific and technical aspects of
217 closed investigations of canine forensics DNA – case series from the University of California,
218 Davis, USA. *Croatian Medical Journal* 52: 280-292. doi: 10.3325/cmj.2011.52.280.

219 Suárez N. M., Betancor E., Fregel R., Pestano J. (2013). Genetic characterization, at the
220 mitochondrial and nuclear DNA levels, of five Canary Island dog breeds. *Animal Genetics* 44:
221 432-441. doi: 10.1111/age.12024

222 Verscheure S., Backeljau T., Desmyter S. (2013). Reviewing population studies for forensic
223 purposes: Dog mitochondrial DNA. *ZooKeys*, 365: 381-411. doi: 10.3897/zookeys.365.5859

224 Webb K. M., Allard M. W. (2009). Identification of Forensically Informative SNPs in the
225 Domestic Dog Mitochondrial Control Region. *Journal of Forensic Sciences* 54: 289-304. doi:
226 10.1111/j.1556-4029.2008.00953.x

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240 **Table 1** Positions of polymorphisms of the 551-bp fragment of mitochondrial DNA control
 241 region in 160 samples of world known dog breeds. Nucleotide positions are numbered
 242 according to the GenBank dog referent sequence, RRS: NC_002008 (Kim et al., 1998) within
 243 haplotype H1. MEDI is present in haplotypes H2, H3, H4, H5, H6 and H7 and they are marked
 244 as bold.

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	1552	1559	1561	1561	1562	1562	1563	1563	1564	1565	1565	1571	1580	1581	1581	1591	1595	1600	1602	1604
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	.	.

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252 **Fig. 1** a) Sampling location: Small Međimurje dog (MEDI), Croatian Shepherd dog (CROS)
253 and Tornjak (TOR); b) Small Međimurje dog male (source: [https://mali-medjimurski-pas-
255 medjimurski-pas-medji.weebly.com/photogallery.html](https://mali-medjimurski-pas-
254 medji.weebly.com/photogallery.html)); c) Small Međimurje dog female (source: [https://mali-
medjimurski-pas-medji.weebly.com/photogallery.html](https://mali-
medjimurski-pas-medji.weebly.com/photogallery.html)).

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

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292 **Fig. 3** Principal component analysis (PCA) determines breed relationships between Small
293 Međimurje dog and groups of dogs based on their utilisation. Hunting group is represented as
294 green dots, Livestock guardian group as cyan dot, MEDI as red dot, Sheepdog group as grey
295 dots and Terrier group as yellow dots. Numbers represent breeds (1 – Croatian Shepherd dog,
296 2 – Small Medimurje dog, 3 – Tornjak, 4 – Airdale Terrier, 5 – Australian Shepherd dog, 6 –
297 Australian Terrier, 7 – Basset Hound, 8 – Belgian Groenendal, 9 – Belgian Malinois, 10 –
298 Beagle, 11 – Border Collie, 12 – Cairn Terrier, 13 – Cocker Spaniel, 14 – Collie, 15 – Corgi,
299 16 – Dachshund, 17 – Dalmatian, 18 – Fox Terrier, 19 – German Bracke, 20 – German
300 Shepherd, 21 – Golden Retriever, 22 – Jack Russel Terrier, 23 – Labrador Retriever, 24 –
301 Manchester Terrier, 25 – Poodle, 26 – Rat Terrier, 27 – Toy Fox Terrier, 28 – West Highland
302 White Terrier, 29 –Yorkshire Terrier, 30 – Cavalier King Charles Spaniel).