

GENETIC CHARACTERIZATION OF NITRA RABBITS AND ZOBOR RABBITS

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ABSTRACT

In an attempt to characterize the two rabbit breeds concerning the shift on the genetic level we studied microsatellite and genetic markers associated with coat color. These two local rabbit breeds, Nitra rabbit (Ni) and Zobor rabbit (Zo) with different phenotypes have a common origin. Based on the highest polymorphic six microsatellites we chose more than 300 rabbits microsatellites. In two analysed experimental groups Ni (n = 8) and Zo (n = 8) the number of detected alleles for six chosen microsatellites was ranged from two to four alleles (4 alleles per INRACCDDV0106, 3 alleles per INRACCDDV0183, 4 alleles per INRACCDDV0259, 4 alleles per Sol08, 2 alleles per SOL28 and 3 alleles per Sol33). Genetic markers were chosen based on the unique characteristics of the two analyzed breeds: mutations c.585delG in the MLPH gene (dilution of coat color), c.5_ginsA in the ASIP gene (characters of wild type rabbit) and c.1118C → A in the tyrosinase gene (albinism). The mutation c.585delG in Ni rabbits is represented only by homozygous genotype del/del. Ni is a rabbit breed with a blue-grey coloring, therefore, in principle, other genotype for c.585delG is not expected in this breed. For Zo breed, only G/G homozygous genotype should be represented, but even in that small group of Zo rabbits (n = 8) one heterozygote del/G has occurred, with the recessive allele hidden under the black phenotype. Concerning ASIP gene, from 8 Ni rabbits 7 animals were homozygous ins/ins, which were blue-coated, and only one was Wt/ins, which was Ni wild-blue, whilst Wt/Wt homozygote was not represented. In Ni and Zo breeds the mutation c.1118C → A in the tyrosinase gene was represented only by CC homozygotes, whereas albinos Hyla hybrid was represented only by AA homozygotes. Here we propose steps to make more efficient the process of cross-breeding of the analyzed Ni and Zo rabbit breeds in order to stabilize and then standardize these two unique rabbit breeds, which could be preserved in the gene bank for possible future use.

Key words: coat color; cross-breeding; genetic marker; microsatellite; rabbit

INTRODUCTION

Rabbit breeds with different names may sometimes have recent common origin, while in other cases their uniqueness has been eroded by cross-breeding. Breeds of rabbits as well as all animals are characterized by the standard of breed, by a set of precisely defined characteristics or parameters established on the basis of phenotypic assessment. The aim of this study is to shift this characterization on the genetic level through a microsatellite and genetic markers associated with coat coloring in two local rabbit breeds with common origin

but so uniquely different phenotype, Nitra rabbit (Ni) and Zobor rabbit (Zo).

Microsatellite markers are useful tool recommended by ISAG / FAO, used for the analysis of genetic diversity in many programs of the livestock preservation (FAO, 2011). Research program ResGen-CT95-60 in 1995-2000 was similarly focused on rabbits in which the European Union was working to inventory, evaluate, conserve and utilize the genetic resources of European rabbits (Bolet *et al.*, 2000), but Slovakia did not participate in this program. For this study, polymorphic rabbit microsatellites

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Sol08, SOL28, Sol33 were chosen from articles written by Rico *et al.* (1994) and SurrIDGE *et al.* (1997) and microsatellites INRACCDDV0106, INRACCDDV0183, INRACCDDV0259 were published by Chantry-Darmon *et al.* (2006).

Genetic markers were chosen base on the unique characteristics of the two analyzed breeds. As a genetic marker for similar study of differentiation analysis, in particular in the case of the definition of genetic resources, traits such as reproduction must not be used such as reproduction characteristics, for which is the effort to achieve in all aspects of their a cost-effectived and balanced level, no go to extremes (Bolet *et al.*, 2000).

In this case it was not possible to evaluate carcasses characteristics, because two similar meat breeds of rabbit were examined. Suitable markers associated with coat color had to be found. Today several genetic mutations associated with coloring are known, from which those were chosen for analysis, which define some of the unique characteristics of these two specific analysed rabbit breeds. The purpose for breeding of these two breeds, Ni and Zo, was to create a white meat rabbit. First, Ni was bred from the cross-breeding of the French silver rabbit with Russian rabbit breed and subsequently with Californian rabbit. Zo breed is the result of cross-breeding of Ni with the New Zealand White rabbit. The uniqueness of Ni and Zo is in specific distribution of pigment areas (color pattern) and their different color. Ni breed is unique by Himalayan color pattern in blue color (nose, ears, feet and tail are blue with clear division between these areas and the white body color), originally in conjunction with characters of wild type rabbit. Ni has clear bright blood red eyes. The Himalayan drawing is typical for Russian rabbit and Californian rabbit, but in black color, while Ni has this color pattern in blue, as previously mentioned. Zo breed has a unique Zobor color pattern (head white, tail black and hindlegs cuffs black with clear division from the white body color). Zo has clear bright blood red eyes, too. Typical Zobor color pattern is a result of combination of Dutch color pattern and partial albinism.

The purpose of this study was the characterization of the unique features of Ni and Zo at the genetic level, which corresponds with genetic markers associated with coat color: mutations c.585delG in the MLPH gene (melanophiline gene) (dilution of coat color, Lehner *et al.*, 2013), c.5_ginsA in ASIP gene (characters of wild type rabbit, Fontanesi *et al.*, 2010) and c.1118C → A in the tyrosinase gene (albinism, Aigner *et al.*, 2000). Epistatic deletion c.585delG in the MLPH gene inhibits the full expression of pigment formation with manifestation of weaker coloring, called dilution

of coat color. Dominantly inherited mutation c.5_ginsA in the ASIP gene A^{Wt} is associated with the characters of wild type rabbit (white inside ears, white underside of the tail, as well as the whole ventral part of the body). Recessive inherited, but also epistatic to all mutation associated with color changes, mutation c.1118C → A in the tyrosinase gene, which in a homozygous recessive line up repressed all pigment production, including the eyes, so-called complete albinism.

We propose steps to make more efficient the process of cross-breeding of analyzed breeds Ni and Zo rabbit to be able to stabilize and then breed-standardize the genetic material of these two unique rabbit breeds, which could be preserved in the gene bank for possible future use.

Ni and Zo are the result of combination of genes of four high quality foreign breeds (Silver French, Russian, California and New Zealand White). In the breed Ni and Zo not only the combination of high quality genetic material is encoded, but at the same time adaptability of this gene combination to our environmental conditions, its efficient use of local natural food sources of forage and its resistance to the pathogenic pressure in our animal production. But choosing the breeds to be evaluated, we did not take into account the risk of extinction of the breed, but their historical status and their potential zootechnical interest (Bolet *et al.*, 2000).

MATERIAL AND METHODS

Animals

The total set of analyzed animals was represented by total of 77 animals from 31 rabbit breeds (Table 4). In this study, three analyzed systems of three rabbit breeds Ni (n = 8), Zo (n = 8) and albinos of hybrid Hyla (n = 3) are considered the key results. From small populations of Ni and Zo rabbits the selection has been made so that the animals conform to the characteristics of the breed standard.

Isolation of DNA

Genomic DNA was extracted from blood using DNA Purification kit Maxwell16[®] (Promega).

Multiplex PCR

The sequence of six polymorphic microsatellite loci was chosen from published articles (Sol08, SOL28 and Sol33 by Rico *et al.*, 1994 and SurrIDGE *et al.*, 1997 and INRACCDDV0106, INRACCDDV0183 and INRACCDDV0259 by Chantry-Darmon *et al.*, (2006), Table 1). Sequences of microsatellites were obtained from GenBank (NCBI). Primers for multiplex PCR were chosen using software QDD2 (MeglécZ *et al.*, 2010).

Table 1: Primer sequences for analysed microsatellite

Microsatellite marker	GenBank. Acc. No	Fluorescence labeling and sequence of forward and reverse primers in 5'-3'	No. of publ. alleles	Reference
INRACCDDV0106	AJ874448	6-FAM: TTGGGAGTGAACCAGCAGAT AATCCGACCCATCTTTCGAT	6	Chantry-Darmon <i>et al.</i> , 2006
INRACCDDV0183	AJ874521	6-FAM: TCACCTTCTACTGCCTGCAC CGCTCTCACTAACTCTGCCTG	7	Chantry-Darmon <i>et al.</i> , 2006
INRACCDDV0259	AJ874589	VIC: CTTTGCATGGGCAATTGAATGCT ACTGAGACAGGGAGGAG	7	Chantry-Darmon <i>et al.</i> , 2006
Sol08	X79217	NED: ATTGGGCCCTTTGCTCACTCGC AGCCATATCTGAGAGAA	1-10	Rico <i>et al.</i> , 1994; Surridge <i>et al.</i> , 1997
Sol28	X79216	PET: AACCACTGACGGAAGTGGAATT ATTATTCAGGGCAGA	1-7	Rico <i>et al.</i> , 1994; Surridge <i>et al.</i> , 1997
Sol33	X94683	6-FAM: CCCATGTGTGCTACTGATTTG GATGTCAAATCTGCCCTCCC	1-15	Rico <i>et al.</i> , 1994; Surridge <i>et al.</i> , 1997

Multiplex PCR conditions and fragment analysis

Multiplex PCR reactions were performed at a total volume of 16 µl using QIAGEN Multiplex PCR Kit (Qiagen), 10-50 ng genomic DNA as template and 0.2 µmol.l⁻¹ of each primer. The PCR profile included an initial denaturation step at 95 °C for 15 min, 28 cycles: 94 °C (60 s), 60 °C (90 s), 72 °C (60 s) and a final extension step of 30 min at 60 °C in C1000 thermal cycler (Bio-Rad). The PCR products were analysed on ABI PRISM 310 and the fragment analysis was performed using the Gene Mapper software 4.0.

PCR

PCR primers sequence for ASIP was taken from Fontanesi *et al.* (2010) and for MPLH from Lehner *et al.* (2013) (Table 2).

STAS-PCR

STAS-PCR for tyrosinase c.1118C-A (T373K) polymorphism. Briefly, two allele-specific internal primers (INT1, INT2) recognized with their 3' nucleotide the allelic variation (Table 2). In order to increase the allele specific annealing an additional mismatch position 3 from the 3' end of each primer was introduced (lower case). The external primers are located at different distances from point mutation, so that different fragment lengths were generated for each allele. In case of nt118C and nt118A homozygotes only one fragment of 79 bp or 99 bp respectively was amplified, whereas in heterozygotes C/A both fragments were detected. Independently of the genotype an additional product (131 bp) of the common external primers (EXT1, EXT2) were amplified.

Table 2: Primer sequences for analysed chosen genetic markers for rabbit coat color

Genetic marker	GenBank Acc. No.	Sequence of primers forward and reverse in 5'-3'	PCR product	Temp. of annealing	Restriction endonuclease
ASIP c.5_6insA	FN547134 Fontanesi <i>et al.</i> , (2010)	CAGGAAGGCACATCCTCTTT GGGTGGCCTGGAGCAGGCGAGTGGAATT	95 bp	57 °C	<i>EcoR</i> I
MPLH c.585delG	KC791692 Lehner <i>et al.</i> , (2013)	GTGAGCAGGCATGTGCAG GGGCCTGAGCAGAGACAC	111 bp	60 °C	<i>HpyF3I</i> (<i>Dde</i> I)
TYR c.1118C→A	AF210660 Aigner <i>et al.</i> , (2000)	EXT1 TGCTAATCCACTTACTGGGATAGC INT 1 GCTTTGCATATGTATATGAATGGcAC INT 2 GATCCCTGTACCTGGGACAcCT EXT 2 CATGGTGAAGGAGAAAAATGGGATC	131 99 79	60 °C	-

The PCR conditions

The PCR reactions were performed at a total volume of 25 µl using 1X Colorless GoTaq Flexi buffer, 2.0 mmol.l⁻¹ MgCl₂, 0.2 mmol.l⁻¹ of each dNTP, 1 U GoTaq G2 Hot Start DNA polymerase (Promega, USA), 50-100 ng genomic DNA as template and 0.4 µmol.l⁻¹ of each primer. The PCR profile included an initial denaturation step at 94 °C for 2 min, 35 cycles of 94 °C (20 s), 57 °C/60 °C (30 s), 72 °C (30 s) and a final extension step of 10 min at 72 °C in C1000 thermal cycler (Bio-rad).

RFLP

The appropriate restriction endonuclease (ThermoScientific, Germany): EcoRI for ASIP and *HpyF3I* (Dde I) for MLPH polymorphism was used to digest PCR products (Table 2).

Microchip electrophoresis of DNA fragments

PCR products were analysed by the automated microchip electrophoresis system MCE[®]-202 MultiNA (Shimadzu, Japan) with a DNA-500 kit according to the manufacturer's protocol. A SYBR Gold fluorescent dye for DNA staining (Invitrogen, USA) and a 25 bp DNA Ladder (Invitrogen, USA) were used to determine the size of the restriction fragments.

RESULTS AND DISCUSSION

The overall population size of Ni and Zo rabbit breeds is very low. Stability of these two breeds is constantly eroded by cross-breeding efforts. Frequent is the occurrence of weak coloring intensity and the lack of compact coloring markings with the appearance of pigment spots on various parts of the body. The selection of the necessary amount of individuals to conform to the breed standard characteristics among them was difficult. However, it 8 specimen of Ni rabbits and 8 specimen of Zo rabbits were selected for the experiment. In order to find genetic variability and to define two breeds on the genetic level, from more than 300 rabbit microsatellites we chose the six highest polymorphic microsatellites. Number of published rabbit microsatellite alleles ranges from 2 to 15 (SurrIDGE *et al.*, 1997). For chosen microsatellites 6 alleles per INRACCDDV0106 (Chantry-Darmon *et al.*, 2006), 7 alleles per INRACCDDV0183 (Chantry-Darmon *et al.*, 2006), 7 alleles per INRACCDDV0259 (Chantry-Darmon *et al.*, 2006), 10 alleles per Sol08 (SurrIDGE *et al.*, 1997), 7 alleles per Sol28 (Rico *et al.*, 1994) and 15 alleles per Sol33 (SurrIDGE *et al.*, 1997) are described.

In our two analysed rabbit experimental groups Ni (n = 8) and Zo (n = 8) the number of detected alleles for six chosen microsatellites ranges from 2 to 4

alleles (4 alleles per INRACCDDV0106, 3 alleles per INRACCDDV0183, 4 alleles per INRACCDDV0259, 4 alleles per Sol08, 2 alleles per SOL28 and 3 alleles per Sol33) (Table 3).

Here we avoided traditional statistical processing, determinations of heterozygosity and genetic distances which would lead to erroneous conclusions rather than to achieve reasonable results, both precisely because of the small numbers of analyzed groups of rabbits, and also because two populations that are genetically distant need not phenotypically differ (Edding and Laval, 1999), and *vice versa*.

Microsatellite is a mean particularly to distinguish individuals under paternity testing. For many species (H cattle, buffalo, sheep, goats, horses, donkeys, camels, pigs and chickens) the sets of microsatellites to identify on the genetic level (FAO 2011) are recommended. For rabbits such microsatellite sets are not recommended yet, although the number of the rabbit microsatellites is described. Since in rabbits very intensive cross-breeding has been done, the origins of the rabbit breeds significantly overlap and this complicates the ability to find appropriate specific microsatellites. Nevertheless, we tried to look for characteristics of these two breeds through microsatellites too. No reallocation alleles in the study of rabbit breeds Ni and Zo were observed as is shown in Table 3. The results of genotyped rabbits for the three genetic mutations associated with change of the coat color c.585delG in MLPH gene, c.5_6insA in ASIP gene and c.1118C→A in tyrosinase gene are listed in the Table 5.

Table 4 shows the results of analysis of mutation c.585delG MLPH in the MLPH gene for 31 rabbit breeds with different coat coloring for a total of 77 animals. This mutation was described by Lehner *et al.* (2013) and Fontanesi *et al.* (2014). Dilution of coat color effect of this mutation was confirmed in breeds Ni, Czech Spot blue, Havana blue, Moravian blue, Gray-blue Rex, Dwarf grey-blue Rex, French Lop blue and Vienna blue (Table 5).

We also thought that coloring in New Zealand red is caused by interaction of the mutation c.585delG in the MLPH gene, too. It is possible however, that in this case the dilution or mixing of pigments is caused by another interactions. One known mutation is now associated with the dilution coat color c.111-5C→A gene MLPH (Lehner *et al.*, 2013) but it was not the subject of the study.

Mutation c.585delG in Ni rabbits is represented only by homozygous genotype del/del. Ni is rabbit breed with blue-grey coloring, therefore, in principle, another genotype for c.585delG is not possible in this breed. On the contrary, it should be for Zo breed, which should be represented only by homozygous genotype G/G for c.585delG, therefore, any likelihood of excision

Table 3: Number of detected alleles for chosen microsatellites in Ni and Zo rabbits

Microsatellite name	Rabbit of Nitra (n = 8)		Zobor rabbit (n = 8)	
	Allele number	Allele size (bp)	Allele number	Allele size (bp)
INRACCDDV0106	4	184_188_190_193	4	184_188_190_193
INRACCDDV 0183	3	152_159_161	2	152_161
INRACCDDV 0259	4	105_116_118_120	4	105_116_118_120
Sol08	4	99_101_104_106	4	99_101_104_106
Sol28	2	100_102	3	96_100_102
Sol33	3	76_79_84	3	76_79_84

Table 4: Results of rabbit genotyping for the c.585 in the MLPH gene

		c.585delG in melanophiline gene		
		del/del	del/G	G/G
Rabbit of Nitra (8)	del/del	8	-	-
Zobor rabbit (8)	del/G, G/G	-	1	7
Californian (8)	G/G	-	-	8
Dwarf Russian (Dwarf Himalayan) (1)	del/G	-	1	-
Czech Spot (5)	G/G	-	-	5
Czech Spot blue (1)	del/del	1	-	-
Czech Spot red (1)	G/G	-	-	1
German Giant Spot (1)	del/G	-	1	-
Spot of Mecklenburg (1)	G/G	-	-	1
Belgian Giant black (2)	G/G	-	-	1
Dutch black (4)	G/G	-	-	4
Havana blue (1)	del/del	1	-	-
Moravian blue (2)	del/del	3	-	-
Grey-blue Rex (2)	del/del	2	-	-
Dwarf grey-blue Rex (1)	del/del	1	-	-
French Lop blue (1)	del/del	1	-	-
French Lop wild type (1)	G/G	-	-	1
Blue of Vienna (2)	del/del	2	-	-
Vienna rabbit (wild coloring) (4)	G/G	-	-	4
European rabbit (3)	G/G	-	-	3
Dwarf european rabbit (1)	del/G	-	1	-
Big Light Silver (3)	G/G	-	-	3
Big Chinchilla (3)	G/G	-	-	3
Rabbit of Rhön (2)	G/G	-	-	2
Dwarf Black (2)	G/G	-	-	2
Dwarf Casto Rex (2)	del/G	-	2	-
Thuringer (1)	del/G	-	1	-
Dwarf Lynx (1)	del/del	1	-	-
New Zealand Red (1)	G/G	-	-	1
Fox-Rabbit white (1)	del/G	-	1	-
Albinos-hybrid HYLA (3)	G/G	-	-	3

Table 5: Results of rabbit genotyping for three analyzed genetic mutations

		c.585delG in melanophilin gene		
		del/del	del/G	G/G
Rabbit of Nitra	8	8	-	-
Zobor rabbit	8	-	1	7
Albinos	3	-	-	3
		c.5_6insA in ASIP gene		
		ins/ins	ins/Wt	Wt/Wt
Rabbit of Nitra	8	7	1	-
Zobor rabbit	8	5	3	-
Albinos	3	-	1	3
		c.1118C→A in TYR gene		
		A/A	A/C	C/C
Rabbit of Nitra	8	-	-	8
Zobor rabbit	8	-	-	8
Albinos	3	3	-	-

offspring with dilution coat color would be eliminated. But even in that small group of Zo rabbits ($n = 8$), one heterozygote del/G is occurred with the recessive allele hidden under the black phenotype. Here is an example, that even if the individual is recognized as a breeding animal, it does not mean that it is „cleans“ (homozygous) for a given reference characters, as many farmers/breeders believe. In case of two heterozygotes del/G for c.585delG in mating plans, their use brings 25 % likelihood of progeny with inadmissible coat color due to dilution. In this way, recognized heterozygotes for c.585delG need not be negatively selected as they will be properly combined with a truly „pure“ homozygote G/G, and all the offspring will have a 100 % full black pigment.

Concerning the ASIP gene, within our small experimental group of 8 pieces Ni 7 rabbits were homozygous ins/ins, these were Ni blue, only one was Wt/ins, and this was Ni wild-blue, whilst homozygote Wt/Wt was not represented. In this case it is necessary to point out one fact when assessing. Ni rabbit breed is bred in two color surges, blue and wild-blue. Blue Ni was bred from wild-blue, Ni blue has intense darker blue-grey color and is without characteristics of wild type rabbit. Currently there is a gradual reduction of wild-blue Ni rabbits, which is the original Ni rabbit with a unique combination of the Himalayan pattern in blue color with wild characters. Modern cross-breeding steps should be reviewed in order to prevent the elimination of unique color lines like that of the wild-blue Ni breed. In relation to the ASIP gene, another major problem was found

in Zo breed too. In our small experimental group ($n = 8$) three Zo rabbits had clear signs of wildness (heterozygotes ins/Wt). Characters of wild type rabbit are inadmissible in Zo rabbit breed, because white hairs move to the pigment black areas. When the allele Wt is present, black characters with white hairs create the effect of less intense coloring. C.5_6insA mutation in the ASIP gene is dominantly inherited and effect of allele Wt is clearly identifiable. According to this it is necessary to put more emphasis on checking wildness in the evaluation of breeding animals of Zo rabbit breed. A clear indicator of the presence of the Wt allele and therefore wildness is the white underside of tail.

Mutation c.1118C→A in the tyrosinase gene, a key enzyme for melanin production, is associated with the expression of complete albinism. In the breeds Ni and Zo mutation c.1118C→A in the tyrosinase gene is represented only by homozygotes CC, whereas albinos Hyla hybrid is represented only by AA homozygote. The results, therefore, confirm the complete suppression of pigment in albino AA assembly, and only homozygous genotypes CC for c.1118C→A in the tyrosinase gene in breeds Ni and Zo. In conjunction with suppression the production of pigment is not specifically defined, concepts/phenomena such as acromelanizmus, partial albinism and leucizmus are misunderstood and often confused, especially in these two breeds. These are therefore excellent experimental material for study and subsequent understanding of these concepts, but this is subject to further studies of the Ni and Zo breeds. Basing on the results of analysis of Ni and Zo rabbit

Table 6: Recommended genetic assembly for three analyzed genetic mutations in two rabbit breeds

	Rabbit of Nitra Wild-blue	Rabbit of Nitra Blue	Zobor rabbit
c.585delG in MLPH gene	del/del	del/del	G/G
c.5_6insA in ASIP gene	Wt/Wt	ins/ins	ins/ins
c.1118C→A in TYR gene	C/C	C/C	C/C

breeds of three systems we propose following genetic assembly shown in the Table 6. For both color surges (blue and wild-blue) of Ni rabbits for c.585delG it should be homozygous assembly del/del, which determines the dilution coat color. For c.585delG of Zo rabbit breed it should be homozygous assembly G/G, ensuring full pigment production on the site-specific staining characters. For c.5_6insA of wild-blue Ni, which has characters of wild-type rabbits as a part of its breeding standard, it should be homozygous assembly Wt/Wt, whilst the ins/ins homozygous assembly is necessary for Blue Ni and Zo, which must be without characters of wild-type rabbit. This system is very effectively monitored thanks to the underside of the tail as effective indicator of wildness (only genotype ins/ins has coloring underside of tail in coloring rabbit breeds, but presence of only one Wt allele is associated with characters of wild-type rabbits). Regarding the last analyzed genetic system TYR_1118, both breeds are important to maintain in the clean homozygous assembly C/C, without the presence of allele A, which needs to be eliminated if clean lines are disrupted by cross-breeding to avoid useless of the negative selection of born albinos. Simply on the basis of the presence of albinos in the litter, without doing genetic analysis, it is possible to confirm whether the parental pair, both male and female, has heterozygous assembly A/C for c.1118C→A in the tyrosinase gene.

CONCLUSION

The implementation of genetic analyses to rabbit breeding is important in order to use all available information and knowledge of similar genetic studies for more efficient breeding techniques to avoid unnecessary false steps, such as the inclusion Marder breed in cross-breeding programs Ni in mistaken belief that the phenomenon acromelanism improves the coloring characteristics of the partial albinism.

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