Animal Science Papers and Reports vol. 32 (2014) no. 2, 121-130
Institute of Genetics and Animal Breeding, Jastrzębiec, Poland

Comparison of different Hungarian Grey herds as based on microsatellite analysis

Attila Zsolnai[1]*, András Kovács[2], István Anton[1], József Rátky[1], Klaus-Peter Brüssow[3], Csilla Józsa[4], Beáta Bán[4], Alice Gyurmán[4]

[1] Research Institute for Animal Breeding and Nutrition, Herceghalom, Hungary

[2] University of Debrecen, Debrecen, Hungary

[3] Leibniz Institute for Farm Animal Biology (FBN), Dummerstorf, Germany

[4] National Food Chain Safety Office, Genetic Laboratory, Budapest, Hungary

(Received April 30, 2013; accepted March 5, 2014)

The present study aimed at assessing the genetic position of the Hungarian Gray population. Hungarian Grey cattle kept at different farms in Hungary have been sampled (34 herds, n=3,187 in the period of 2009-2011) to investigate their genetic relationship based on analysis of allelic variation at eleven microsatellite *loci*. The mean observed heterozygosities per herd were above a moderate degree (0.60-0.80). Calculation of pairwise genetic distances and analysis of the history of herds revealed that among the most closely related herds we can find those, which are the core of the current Hungarian Gray population. The results of the population differentiation showed that all Hungarian Gray herds were significantly different from each other. In most cases (22 herds) F_{ST} values were within a range of low degree of genetic differentiation (0.003-0.050), while the remaining 12 herds differed from the central population by F_{ST} values of 0.060-0.119. Principal coordinate analysis, assignment tests and dendrograms all suggest that there are mainly two different groups among Hungarian Grey herds. Structure analysis has yielded K=3 as the most probable number of clusters. Based on the private allelic richness, genetic distance and F_{IS} values identified were 12 herds where more attention should be paid by the management to avoid genetic drift and to preserve genetic diversity. The results presented could also contribute to the proper selection of animals for further whole genome scan studies of Hungarian Grey.

KEY WORDS: cattle / genetic distance / herds / Hungarian Grey cattle / microsatellite

*Corresponding author: attila.zsolnai@gmail.com

The first description where long-horned cattle "*magnus cornuotes boves Hungaricos*" can be recognised as Hungarian Grey (HG) was found in a 16th-century document [Milhoffer 1904 cit. Bartosiewicz 1997]. Before this time (10th-13th century) most cattle in Hungary had been small, brachyceros type animals [Bartosiewicz 1997]. The long-horned larger cattle became common in 16th-18th century, *i.e*. during the Turkish occupation [Bartosiewicz 1996, 1997]. In 1884, 78% of the 4.9 million cattle were registered as HG and half of the 6.7 million belonged to this breed in 1900 [Mattesz 1927, Tormay 1901, both cit. Bartosiewicz 1997]. In 1925 there were 321,000 HG cattle in the remaining central part of the former Hungary, and in 1962 only 200 purebred cows and six bulls were saved [Bodó *et al*. 1996]. The rescue program initiated by Imre Bodó and supported by the Hungarian government started in 1961. At the present time the HG population amounts to 7,000 cows [Bodó 2011].

The HG belongs to the "Podolic" cattle group named also Podolian or West-Ukrainian however, the origin of its members has been questioned by several authors [Maróti-Agóts 2011, Manzone 2011, Filippini 2011]. Phenotypic traits like strong pigmentation, long dark eyelashes and well developed dewlap point to its southern origin showing similarities to bequeathed, thousand years old pictures from Egypt and Sahara region. The Turkish Ottoman Empire occupied North-Africa, including Egypt and the Near-East before the Balkan countries and Central-Hungary, so cattle of African and Near-East origin could enter Europe accompanying the Turkish army in the 16th century. However, results of mitochondrial DNA studies in HG and Italian Grey Cattle breeds showed similarities to other European breeds, and the T1 haplogroup, located phylogeographically in Africa, was not observed in HG cattle [Maróti-Agóts 2011].

A few Maremmana bulls were brought to Hungary in the early 20th century and another three in 1971 [Bodó 2011], the hereditary 1;29 chromosome translocation was introduced by one of them. This abnormality which is linked to embryo losses was not found in purebred HG, and it was eradicated from the affected non-HG herds by investigating more than 800 cattle and culling the carriers of both sexes, respectively [Kovács 1989].

Microsatellites are widely used to characterise of populations and have already been applied in genetic analysis of 60 HG individuals [Manatrinon *et al*. 2008]. Furthermore, 91 SNPs have been identified from 63 HG individuals [Pariset *et al*. 2010]. In both studies HG cattle were compared to several other local breeds including Maremmana.

The present study aimed at assessing the genetic position of the HG herds. The assessing enables: (1) identification of herds which are distinct from the core population, so that the breeding associations and managements could pay more attention to their breeding decisions, and (2) selection of individuals for SNP-chip genotyping and whole genome sequencing, which truly represent the genetic background of HG. Similar approach for revealing breed specific, trademark-like sequences started with microsatellite analysis has already been proved successful in Mangalica breed of pigs [Zsolnai *et al*. 2006, 2013, Szántó-Egész *et al.*2013].

Material and methods

Samples and microsatellites

The registry of herds referring to the codes used herein is available for the breeders and researchers in the National Food Chain Safety Office, Genetic Laboratory, Budapest, Hungary. Herd locations have been revealed to the researchers after statistical assessment of genetic data.

HG animals (n=3,187, Tab. 1) were being selected from year 2009 to 2011. Farms that submitted more than 25 samples were chosen for further study. Maremmana samples from Italy (MI, n=52) were also included as a reference group.

In total, polymorphism at eleven microsatellite *loci* was analysed. All microsatellite markers used are recommended by International Society of Animal Genetics (ISAG) for routine parentage control and record exchange between laboratories [ISAG species panel 2003, http://www.isag.us/comptest.asp?autotry=true&ULnotkn=true]. QIAamp DNA Mini Kit (QIAGEN, US) was used for DNA preparations. PCR conditions were applied according to instruction manual of Bovine Genotypes Panel 1.1 (Finnzyme Diagnostics, Finland), whereas the PCRs were performed on an ABI 9700 (Applied Biosystems, US). Fragment length determination was accomplished on ABI 3100 Genetic Analyzer (Applied Biosystems, US) according to the manufacturer's instructions.

Data analysis

Exact test of Hardy-Weinberg equilibrium and exact test of population differentiation were calculated by Genepop 4.2.1 [Rouset 2008], FSTAT 2.9.3.2 [Goudet 2005] and Arlequine [Excoffier *et al*. 2005] programmes. Estimation of exact P value of Hardy-Weinberg test was performed on each *locus* and each population. Evidence for the presence of null alleles at each *locus* was evaluated using Genepop and Micro-Checker version 2.2.3 (Monte Carlo simulation; bootstrap method) [Van Osterhout *et al*. 2004]. Ho, He, F_{IS}, F_{ST}, N_m indices were calculated by Genalex 6.5 [Peakall and Smouse 2006]. Data were crosschecked by FSTAT and Genepop. F_{IS} values were standardized for the population sizes using weighted means in comparison between the populations (Tamhane's test, SPSS for Windows). Allelic richness (AR) and private allelic richness (AR_P) were calculated with rarefaction procedure by software HP-Rare 1.0 [Kalinowski 2005]. For pairwise F_{ST} (Analysis of Molecular Variance function, permutation: 999) and Nei's genetic distance calculations Genalex was applied, F_{ST} matrix was submitted to Principal Coordinate Analysis (PCoA) in Genalex. Nei's genetic distance (D_A) was also calculated and viewed by TFPGA [Miller 1997] and Poptree [Takezaki *et al*. 2010] softwares. Bootstrap values were based on 1000 permutations.

Bayesian algorithm implemented in Structure was used for inferring the most probable number of clusters (K) (burn-in: 10^5, MCM steps: 10^5, repetition: 10, model: admixture, allele frequencies correlated) and for calculation of membership

probability of individuals. For estimation of K, Evanno's method [Evanno *et al*. 2005] was applied on Structure output. Bayesian stochastic partition-based approach implemented in BAPS 6.0 [Corander *et al*. 2003] has also been applied to estimate K and to assess degree of proportion of MI in HG animals.

Assignment tests of the individuals was performed by Genalex and Geneclass 2.0 [Piry *et al*. 2004] using a Bayesian method [Rannala and Mountain 1997] and a simulation algorithm [Paetkau *et al*. 2004] with 10 000 simulated individuals.

Results and discussion

Null alleles were not detected in analysed microsatellite markers. Altogether, 72 of the 374 chi-square tests showed significant deviations from Hardy-Weinberg equilibrium (HWE) at the 95% confidence interval. Heterozygote excess was significant ($P<0.05$) in herds G, I, J, N, P, R and AA-AK populations except AD and AI. Homozygous excess was significant in herds A, C, L, Q and Z.

Herds A-Z had their F_{IS} values slightly below zero, while heterozygote excess (Tab 1.) could be seen in the AA-AK populations. These could result of possible outbreeding in the past or more likely the consequence of breeding policy, where more distant animals are selected for producing offspring.

Significant differences in F_{IS} values were found in AE, AH and AJ herds against the majority of other herds (data not shown). Private alleles were detected in 11 herds, including those which are genetically more distant from population A-Y. Herds A, C and AI harboured four private alleles each; whereas herd B and Z both had six-private alleles on different *loci*. Herds A, B, C, Z and AI indicate their importance in keeping the genetic diversity in Hungarian Greys. Estimated allelic richness of private alleles, which were calculated by HPRare software, showed decreasing trend starting from herd A to herd Q (Tab. 1.). However, herds Z and AI had the highest rarefied private allelic richness (0.39 and 0.32), AK and AB are at the eighth and 9th position with values 0.06 and 0.03, respectively. With regard of allelic richness, herds A, B and F had higher values (6.87, 6.11, 6.01 respectively), while the lowest allelic richness was detected in herds AA, AF, AG, AK and AH (AR: 4.99-4.49).

Pairwise exact genotypic differentiation tests performed by FSTAT and Genepop showed that all the herds, but four herd pairs (H *vs*. Q, X *vs*. Q, X *vs*. C and X *vs*. T that were all located in close geographical proximity), can be treated as a separate units, distinct from each other ($P<0.05$).

Consecutive PCoA analysis of estimated F_{ST} values ($P<0.000$) revealed 12 herds (Z and AA-AK) distinct from the majority of other HG herds (Fig. 1). The pairwise F_{ST} values of these 12 populations compared to the others (0.060-0.119) fell into the range of moderate genetic distance: 0.050-0.150 [Hartl and Clark 1997]. The genetic differentiation (F_{ST}) among the remaining 22 populations was in the range of 0.003 and 0.05. Only M-T pair fell out of this range ($F_{ST}=0.061$). The pairwise F_{ST} values obtained among herds have been also deposited in the Genetic Laboratory of the

Table 1. Diversity statistic (±SE) for the Hungarian Grey cattle

Herd code	N	AR	AR_P	H_o	H_e	F_{IS}
A	120	6.87	0.25	0.68 ±0.03	0.71 ±0.02	0.035 ±0.04
B	142	6.11	0.11	0.74 ±0.03	0.73 ±0.03	-0.017 ±0.01
C	134	5.88	0.12	0.69 ±0.03	0.69 ±0.02	0.004 ±0.02
D	145	5.85	0.07	0.71 ±0.02	0.69 ±0.02	-0.024 ±0.02
E	123	5.46	0	0.70 ±0.04	0.69 ±0.03	-0.024 ±0.02
F	153	6.01	0.02	0.70 ±0.03	0.69 ±0.03	-0.018 ±0.01
G	97	5.98	0.11	0.75 ±0.03	0.73 ±0.03	-0.036 ±0.02
H	149	5.83	0.02	0.71 ±0.02	0.70 ±0.02	-0.023 ±0.01
I	131	5.82	0.02	0.73 ±0.04	0.70 ±0.03	-0.035 ±0.01*
J	53	5.79	0	0.71 ±0.04	0.67 ±0.04	-0.057 ±0.02
K	117	5.74	0	0.70 ±0.04	0.69 ±0.04	-0.021 ±0.01
L	118	5.59	0.01	0.68 ±0.04	0.70 ±0.02	0.020 ±0.04
M	40	5.35	0	0.70 ±0.03	0.67 ±0.02	-0.050 ±0.03
N	115	5.31	0.01	0.69 ±0.06	0.65 ±0.05	-0.053 ±0.02
O	55	5.09	0	0.70 ±0.04	0.68 ±0.04	-0.032 ±0.02
P	169	5.51	0	0.73 ±0.02	0.70 ±0.02	-0.048 ±0.01*
Q	123	5.77	0.01	0.66 ±0.03	0.70 ±0.02	0.054 ±0.03
R	150	5.93	0.02	0.72 ±0.03	0.69 ±0.03	-0.034 ±0.01
S	157	5.79	0	0.72 ±0.03	0.71 ±0.03	-0.009 ±0.02
T	102	5.48	0	0.70 ±0.03	0.68 ±0.03	-0.032 ±0.02
X	105	5.87	0.02	0.69 ±0.03	0.69 ±0.03	-0.002 ±0.02
Y	61	5.65	0	0.73 ±0.03	0.69 ±0.02	-0.058 ±0.01*
Z	50	5.70	0.39	0.60 ±0.06	0.64 ±0.06	0.042 ±0.05
AA	38	4.99	0	0.70 ±0.03	0.66 ±0.02	-0.067 ±0.03
AB	158	5.40	0.03	0.71 ±0.02	0.67 ±0.03	-0.060 ±0.02
AC	46	5.47	0	0.72 ±0.03	0.64 ±0.03	-0.133 ±0.03*
AD	35	5.49	0	0.69 ±0.05	0.65 ±0.04	-0.069 ±0.04
AE	28	5.36	0	0.71 ±0.05	0.61 ±0.05	-0.181 ±0.03
AF	29	4.96	0.02	0.63 ±0.04	0.57 ±0.03	-0.100 ±0.04
AG	34	4.96	0	0.73 ±0.04	0.67 ±0.04	-0.096 ±0.02*
AH	55	4.49	0	0.80 ±0.03	0.66 ±0.02	-0.205 ±0.02*
AI	55	5.32	0.32	0.67 ±0.05	0.64 ±0.05	-0.048 ±0.03
AJ	51	5.01	0	0.76 ±0.04	0.66 ±0.03	-0.147 ±0.02*
AK	49	4.69	0.06	0.70 ±0.03	0.65 ±0.02	-0.079 ±0.03

*F_{IS} – inbreeding coefficient values did not differ significantly from zero after bootstrapping (C.I.=0.95).
n – number of individuals; AR – allelic richness; AR_p – rarefied private allelic richness; H_0 – average observed heterosygosity (mean±SE); H_e – average expected heterosygosity (mean±SE).

Hungarian National Food Chain Safety Office. The F_{ST} values between MI and A-Y and between MI and Z-AK groups were 0.090 and 0.100 respectively, indicating moderate genetic differentiation. $F_{ST\ (Z\text{-}MI)}$ value was higher (0.169) which was in the very great differentiation range. Herd Z was genetically closest to the two founder populations; herds A and B ($F_{ST\ (Z\text{-}A)}$=0.086, $F_{ST\ (Z\text{-}B)}$=0.086).

The dendrogram of Nei's genetic distance (Fig. 2.) of populations showed two distinct groups; the circled branches represent those herds, which are genetically less divergent from each other. HG herds inside the circle were the same previously identified by PCoA analysis (blue, unlabelled herds on Fig. 1.).

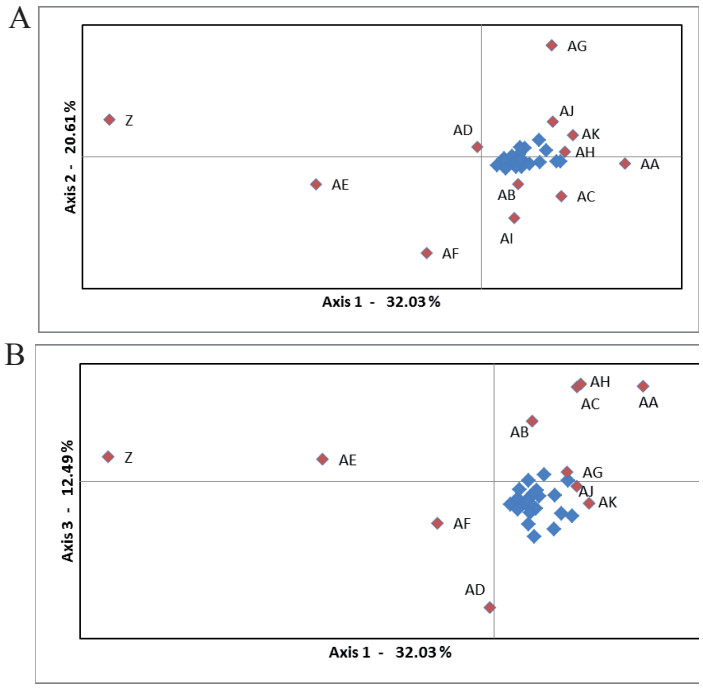

Fig. 1. Representation of PCoA of estimated pairwise F_{ST} values obtained by Genalex software. Blue unlabelled herds (which are from herds A-Y) are in the range of low genetic differentiation. Red labelled herds (Z-AK) have moderate genetic distance from herds A-Y. Percentage values represent variation justified by each axis.

In genetic assignment test (data not shown) only 11-59 % of the animals have been allocated correctly to their original groups in herds A-Y, meanwhile the corresponding values were 69-100% in the remaining herds (Z-AK), supporting that herds A-Y are more closely related than Z-AK to each other or to A-Y.

Structure programme revealed that the most probable number of clusters among 34 HG herds was 3 (K_{Evanno}=3, Fig. 3.). K value was 14 when the lowest mean posterior probability (lnP(D) = -102716, $K_{lowest_p_prob=}$14) was choosen as a criterion. The most probable number of clusters was also determined for A-Y (22 populations) and Z-AK (12 populations) separately. The value obtained by K_{Evanno} method was 3 at each case. $K_{lowest\_p\_prob}$ has obtained value of 8 and 9, respectively. Clustering of populations determined for 34 (A-AK), 22 (A-Y) and 12 (Z-AK) HG populations showed K=18 (Fig. 4.), K=9 and K=9 calculated by BAPS software, respectively. From the history of herds it is known that at the time of bottleneck events around the year 1960 the number of founder populations of current herds was 3, which is supported by K_{Evanno}=3 values.

The existence of 12 outlier populations (Z, AA-AK) among Hungarian Greys may imply genetic drift, isolation, or possible introgression of other breeds in these herds in the past. Isolation has happened in herd Z that this herd has been kept inaccessible

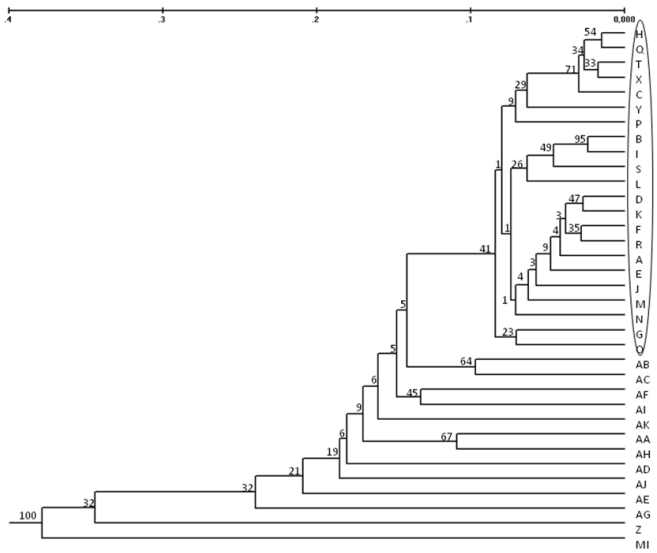

Fig. 2. UPGMA tree of Nei's genetic distance of Hungarian Grey from 34 farms and Maremmana samples (MI). Members of the circled group have low genetic differentiation between each other. Bootstrap values are indicated on the nodes.

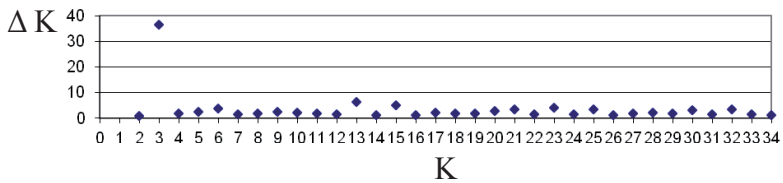

Fig. 3. Determination of the most probable cluster number of 34 Hungarian Grey herds using Δ K approach on Structure lnP(D) values. Δ K values (ten independent runs) for each assessed *K* value on 34 HG populations. The most probable number of clusters was three.

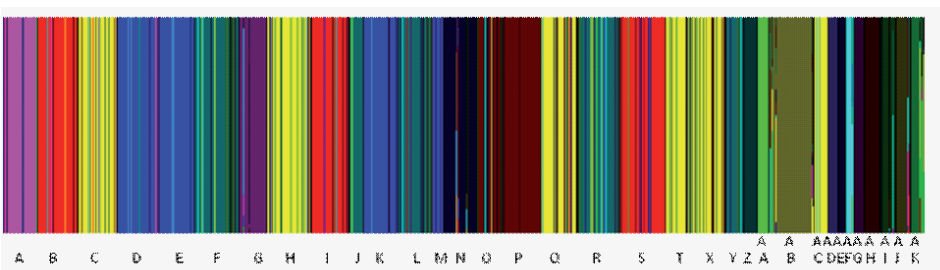

Fig. 4. Clustering of groups of Hungarian Grey individuals (displayed as vertical lines) by BAPS software. Clusters where more then one groups are belonging to: B, I, S (red), C, H, Q, T, X, AC (yellow), D,E,K,M (blue), O, P (braun) F, J, L, R, Y (green), N, AE (deep blue).

to the other herds for about hundred years. According to their documentation farms AD, AJ and AK have used animals of different origin (breeds), so their positions are not surprising. After our sampling period, all HG animals from farm AK have been sold for food processing. Herd AG is known to have well documented breeding scheme (*i.e.* no other than HG breed is applied in breeding), but its distinctiveness might reflect an accumulating genetic drift.

The position of MI in phylogenetic tree (Fig. 2.) provides evidence, that Maremmana cattle had little or no impact on the HG population [Kovács 1989]. In this study most HG individuals have never been placed to the MI cluster in assignment tests. Our present results are in accordance with Pariset *et al*. [2010], where similarity between the two breeds (HG-MI) was detected only in phenotypes. However, using Genalex and GeneClass, two and five animals (0.06-0.16% of the studied HG population) were assigned to MI breed, respectively. Moreover, structure software assigned 69 HG animals (2.13%) to MI group with probability up to 0.346 of having ancestral origin from MI breed. Using option of admixture analysis in BAPS software we have identified 10 HG animals fallen into MI cluster, possessing 0.50-0.67 membership coefficient with probabilities of 0.080-0.340. For obtaining more accurate information about the proportion of Maremmana breed in these animals SNP markers should be used. These markers should be carefully selected from whole genome scan data (using e.g. Illumina BovineSNP50 Genotyping Beadchip) as Fkronja *et al.* [2012] demonstrated in case of composite Swiss Fleckvieh cattle.

Pairwise F_{ST} value obtained herein (F_{ST}=0.123, 52 MI individuals *vs*. 2690 animals from 34 HG farms) was similar to the SNP markers based value of 0.124 calculated by Pariset *et al*. [2010] using 93 MI *vs*. 63 HG samples. F_{IS} value was a small positive number (0.031) in the paper of Manatrinon *et al*. [2008], where 60 Hungarian Greys were sampled. F_{IS} value of -0.048 reported by Pariset *et al*. [2010] was also in good accordance with our microsatellite based F_{IS} value (-0.047). The small negative F_{IS} (-0.047) and the estimated gene flow (the number of migrant animals, Nm=8.536) indicate good calculated mating scheme used in HG population.

Takács *et al*. [2006] compared Nei's genetic distance of five HG populations using the same microsatellite set as applied in this study. Three out of these five populations were also included in our study (herd A, B and C). Regarding the Nei's genetic distances (D_A, 1983) among A, B and C we noticed slightly different values compared to Takács *et al*. (2006) (differences were +0.019, -0.003, -0.028, in case of A-B, A-C and B-C pairs, respectively). These differences might reflect the effect of using different breeding bulls at different sampling periods.

In summary, identified were 12 Hungarian Grey herds, from which animals should be used cautiously in an initial whole genome population study, to avoid confusing results. We have also identified herds, which we regarded important in maintaining the overall genetic diversity of this breed. The present study is providing assistance for further investigations and can contribute to the proper allocation of financial and scientific resources [Boettcher *et al*. 2010] to maintain herds efficiently.

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