ORIGINAL PAPER

Genetic structure of *Curculio elephas* Gyll. (Coleoptera, Curculionidae) in Greece: an important pest of sweet chestnut

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Abstract The weevil, *Curculio elephas* (Coleoptera, Curculionidae), is one of the most abundant and widespread chestnut feeding pests in Greece. Together with the tortricid moths *Cydia splendana* and *C. fagiglandana* (Lepidoptera, Tortricidae), *C. elephas* greatly affect chestnut production. This weevil occurs in a continuous range throughout Greece; however, its population structure is unknown. Information concerning its population structure could help in the future control of this pest. A 790 bp portion of the mitochondrial *Cytochrome Oxidase I* gene was sequenced for 160 *C. elephas* larvae from ten populations. Compared to other *Curculio* species haplotype diversity with 31 haplotypes and nucleotide diversity with six nucleotide substitutions was low. The Bayesian inference of phylogeography in concert with nested clade analysis revealed a loose geographic distribution of genetic diversity that was likely the joint effect of isolation by distance and gene flow. In addition, population indices and Bayesian-based calculations showed that the populations of *C. elephas* in Greece are at the phase of expansion. Dating the phylogeny suggests about 300,000 years before present as the start of divergence among the populations of *C. elephas* in Greece.

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Introduction

Sweet chestnut, *Castanea sativa* Mill., is one of the most common trees of the Mediterranean flora and first acknowledged in human history between 10,600 to 8,900 years before present (Yasuda et al. 2000; Marinova et al. 2002). Since then, the interest in chestnut cultivation fluctuated with evidence of periods of intense use of chestnuts followed by periods of little use (Pite 1986; Santos et al. 2000; Conedera and Krebs 2008). Before the end of the last century, interest in chestnut cultivation increased. This is an economically promising industry and chestnut cultivation exhibits a potential that is not fully realized (Conedera and Krebs 2008; Diamandis 2008). The revitalization in chestnut cultivation is reflected in the increase of studies concerning *C. sativa*, which address the benefits of its wood and seeds as well as to its diseases and pests (Anagnostakis 1988; Paparati and Speranza 1999; Perlerou and Diamandis 2006). The biology, ethology and distribution of chestnut pests in Europe, and particularly those that feed in the nut have been extensively studied because they can greatly decrease chestnut production (Menu 1993; Menu and Debouzie 1995; Csóka 1997; Desouhant 1998; Speranza 1999; Hirka 2003; Avtzis 2012; Avtzis et al. 2012). The important weevil pest, *Curculio elephas,* was found to infest 35 % of chestnuts in Greece in 2012 (Avtzis et al. 2012).

Curculio elephas occurs in a continuous range throughout Greece including its islands (Avtzis et al. 2012). The interspecific relationships and movement of the weevil among locations is unknown. Phylogeographic

results, including demographic, historical and population parameters, can be used to estimate the current status and future potential of a pest (Avise 1998). This information can be integrated into a pest management strategy. These results and conclusions rely on a robust estimate phylogeny which considers taxon sampling and phylogenetic methodology. The chestnut weevil has been included in a phylogenetic study that investigated the interspecific relationships among *Curculio* species, however, conclusions concerning their population genetics were not (Hughes and Vogler 2004).

This study endeavors to resolve the population structure of *C. elephas* in Greece, and the factors that contributed to the contemporary distribution of genetic diversity. We use Bayesian phylogenetic inference and other phylogenetic software to show that past geographic isolation and contemporary geneflow has influenced the population genetic structure of *C. elephas* in Greece.

Materials and methods

Chestnuts were manually opened and all living larvae were placed in individual vials with 100 % ethanol. We collected 160 *C. elephas* larvae from ten populations for this study (Table 1; Fig. 1). DNA from each larva was extracted using GenElute Kit (Sigma) and processed following the manufacturer's protocol. Amplification of an ~800 bp locus from the 3′ end of mitochondrial *Cytochrome Oxidase I* (COI) gene was carried out with the primers Jerry and Pat (Simon et al. 1994) in 25 µl reactions containing 0.6 µl (3 u) of MyTaq™ (BioLine, GmBH, Germany), 5 µl of the provided 5× MyTaq™ red reaction buffer (BioLine, GmBH, Germany), 0.75 µl of each primer (20 µM), 8 µl of DNA extract and ddH$_2$O to the volume of 25 µl. PCR conditions included an initial denaturation step at 94 °C for 3 min, followed by 40 cycles at 94 for 30 s, 45 for 30 s, and 72 °C for 1 min with a final extension step that lasted 5 min at 72 °C. PCR products were then purified with PureLink™ PCR Purification Kit (Invitrogen) and sequenced with an ABI 3730XL at CEMIA SA (Larissa, Greece) using both primers.

Data analysis

Sequences were visualized using CHROMAS v. 1.45 and then aligned using CLUSTAL X (Thompson et al. 1997) with the default settings. After haplotypes were identified, and to exclude cases of base misincorporation as a result of PCR error (Kobayashi et al. 1999), those represented by only a single individual were verified by additional sequencing of an independent amplicon. The sequences of all *C. elephas*

Table 1 Populations, individuals, and haplotype diversity per population of *Curculio elephas* in Greece

	Population	No. ind. analyzed	Haplotype div.
1	Aghio Oros—Halkidiki	10	0.40000
2	Eastern Crete	23	0.08695
3	Hortiatis—Thessaloniki	3	0.33000
4	Melivia—Larissa	51	0.19607
5	Aghiasos—Lesvos	8	0.37500
6	Halkida—Evia	16	0.43750
7	Western Crete	25	0.20000
8	Kastri—Lakonia	1	1.00000
9	Paiko—Pella	3	0.33000
10	Amea—Halkidiki	20	0.40000
	Total	160	

and *C. glandium* (outgroup) haplotypes were deposited in GenBank (Accession Nos: KC663584–KC663614), and referenced by haplotype designations provided below. Bayesian-based inference was performed with MRBAYES version 3.1.1 (Ronquist and Huelsenbeck 2003) using the nucleotide substitution model F81 (Felsenstein 1981) as suggested by MRMODELTEST v2.1 (Nylander et al. 2004). However, prior fixed values for rates and nucleotide frequencies were not used as recommended (Ronquist et al. 2011). The sequence of *C. glandium* was used as outgroup in the above-mentioned analysis. The number of generations was initially set to 5,000,000 with a sampling frequency of 100 generations in a dual run each with four chains. After 1,500,000 generations, stationarity was achieved with the average standard deviation of split frequencies ranging between 0.00298 and 0.00191. Therefore, the last 8,500 trees of each run were used to compute the 50 % majority rule consensus tree and posterior probabilities. The frequency and spatial distribution of the mitochondrial DNA haplotypes were used to test the null hypothesis of random association of maternal lineages with geographic locations. A statistical parsimony network (Templeton et al. 1992) was created using TCS (Clement et al. 2000) and no ambiguous loops were detected. Haplotypes were then clustered into a hierarchical series of groups (Templeton et al. 1992) and the resulting nested network was statistically evaluated with GEODIS v. 2.0 (Posada et al. 2000).

Patterns of molecular diversity based on the mitochondrial DNA sequences between and within populations were assessed by estimating haplotype and nucleotide diversity (π) (Nei 1987) and the average number of nucleotide differences (Tajima 1983) using the software DNASP version 5 (Rozas and Rozas 1999). Isolation by distance was evaluated by Mantel test (Mantel 1967), as this is inferred in the program MANTEL NON-PARAMETRIC CALCULATOR

Fig. 1 **a** Distribution of collected *C. elephas* populations in Greece. Circles indicate the phylogenetic clades and the remaining haplotype as shown in (b). **b** 50 % majority rule consensus tree derived from the Bayesian analysis of *C. elephas* mitochondrial haplotypes. Numbers above branches indicate Bayesian posterior probabilities (>0.85). **c** Nested Clade Analysis of *C. elephas* mitochondrial DNA haplotypes. Empty circles indicate missing haplotypes whereas the size of each parallelogram is proportional to the individuals it represents. *Color* of clade is common in all three figures. (Color figure online)
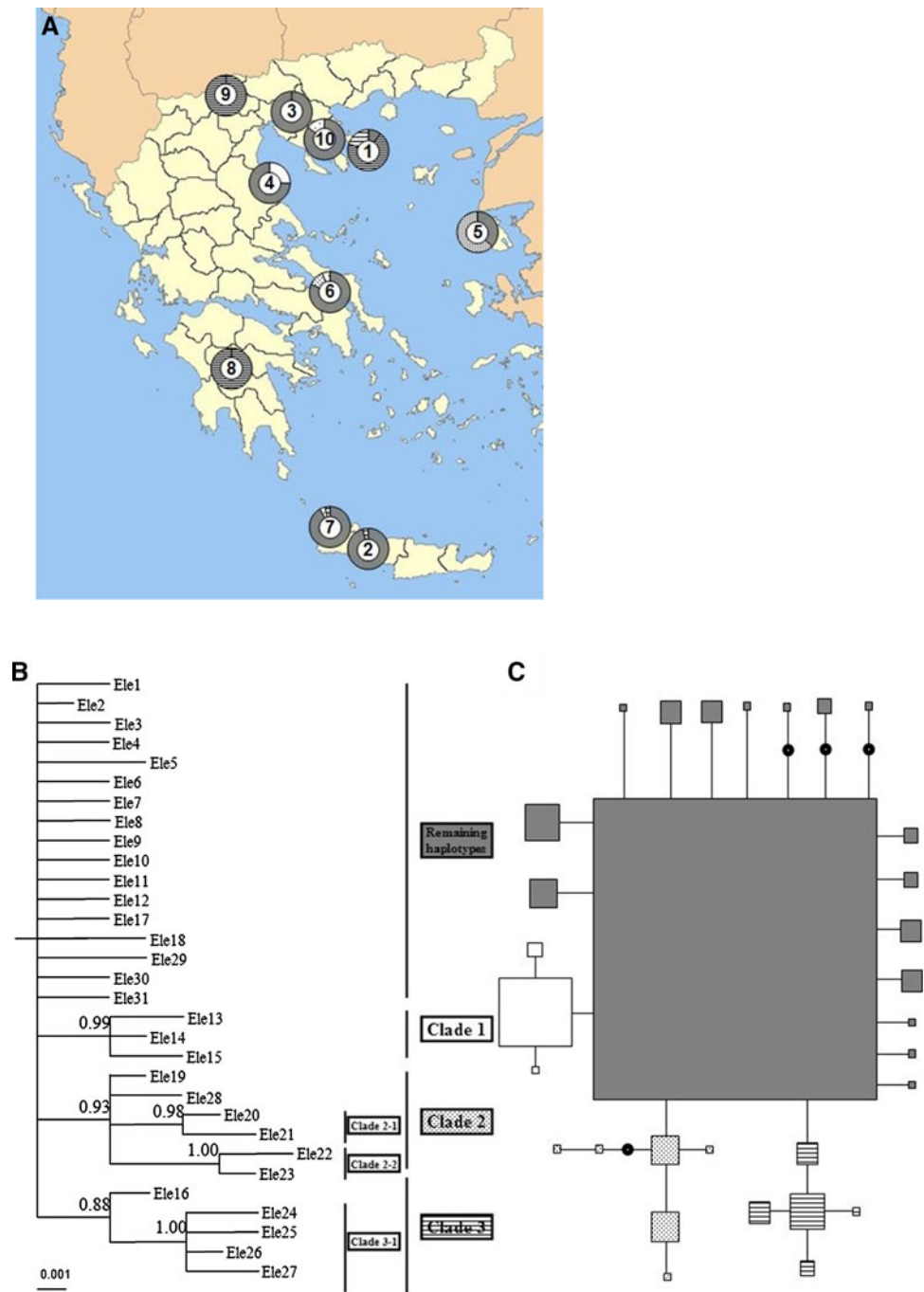
VERSION 2.0 (Liedloff 1999). Natural logarithms of geographical linear distances (km) between localities were correlated with the respective Tamura and Nei (1993) genetic distances, calculated with MEGA v.5 (Tamura et al. 2011), with 1,000 random iterations to obtain statistical inferences at $\alpha = 1 \%$. Sequence data were also examined for evidence of demographic expansion using two approaches: (i) mismatch distribution and (ii) Tajima's *D* statistics (Tajima 1989). When at equilibrium, the

distribution of pairwise differences is expected to yield a multimodal distribution, whereas sudden demographic expansion is commonly associated with unimodal distributions (Slatkin and Hudson 1991; Rogers and Harpending 1992). In addition, Tajima's *D* statistics were used as a complementary approach to investigate the demographic history of the species. Both mismatch distribution among haplotypes (with 1,000 randomizations) and Tajima's *D* were calculated using DNAsp version 5 (Rozas and

Rozas 1999). In addition, the demographic history of *C. elephas* in Greece was assessed through a lineage through time (LTT) plot, as this is implemented in the Bayesian phylogenetic software Beast (Drummond and Rambaut 2007). Finally, an analysis of molecular variance (AMOVA) was used to examine the amount of genetic variability partitioned within and among populations as well as among groups of populations with Arlequin ver.3.0 (Excoffier et al. 2005). To elucidate the contribution of different factors, populations were clustered according to geographic distribution using a non-parametric approach (Excoffier et al. 1992). In addition, a spatial analysis of molecular variance was used to define the groups of populations using SAMOVA 1.0 (Dupanloup et al. 2002). This software seeks the composition of a user-defined number (*K*) of groups of geographic adjacent populations that maximize Φ_{CT}. The program was run for 10,000 iterations for *K* = 2 to 9 from each of 100 random initial conditions.

Estimations of divergence times on the COI locus among the major phylogenetic clades of our analyses were carried out with the Bayesian inference method implemented in MDIV software (Nielsen and Wakeley 2001). Using a Markov Chain Monte Carlo simulation of the coalescence process in concert with the HKY model of sequence evolution to correct for multiple substitutions, we generated maximum likelihood estimators for the population parameter theta θ ($\theta = 2N_e\mu$), the migration rate between populations since divergence *M* ($M = 2N_e m$) as well as the time since divergence in mutational time *T* ($T = t_{div}/2N_e$), with N_e being the female haploid effective size, μ the per locus mutation rate, *m* the migration rate and *t* the number of years since divergence. Initially the method was tested for different values of *M* (1, 3, 5, 10 and 20) and *T* (5, 10, 15 and 20); only then did we set our prior values for *M* = 10 and *T* = 20, as they produced consistent posterior distributions. MDIV analyses were run for 5×10^6 generations with a burn-in period of 5×10^5, and repeated three times to insure convergence. The coalescent-scaled parameter *T* was converted to number of years since divergence t_{div} according to the formula $t_{div} = T(\theta)/2\mu$. For the calculation of μ, it was assumed one generation of *C. elephas* per year and a neutral rate of 2 % per million years (Papadopoulou et al. 2010), and thus for the 790 bp long locus μ was 7.9×10^{-6} substitutions/site/year.

Results

A total of 31 mitochondrial DNA COI haplotypes were found among 160 *C. elephas* individuals (Table 1; Fig. 1a). Haplotype diversity was 0.7041 (±0.0405), whereas nucleotide diversity (π) among all haplotypes was 0.001668 (±0.001157) (Table 2). Mean number of
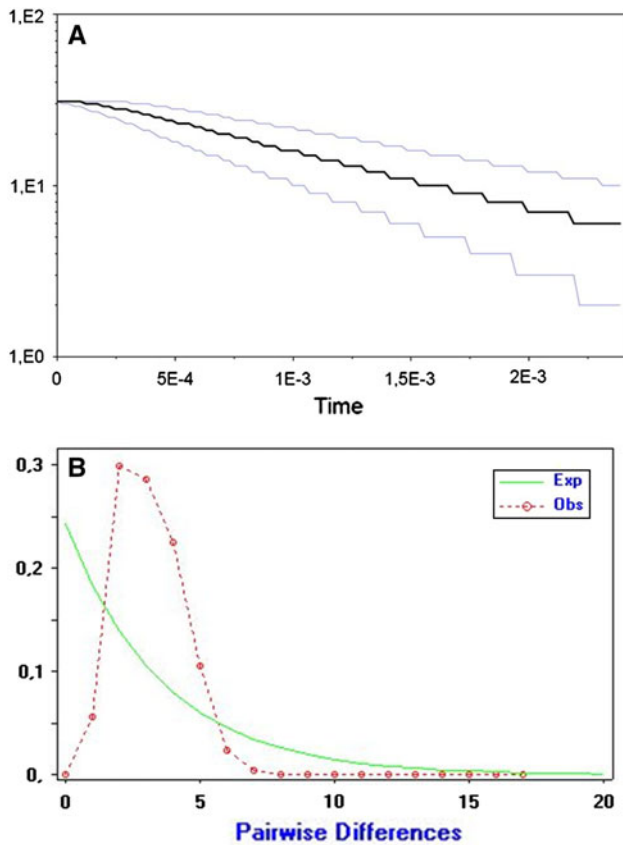
pairwise nucleotide differences among all individuals was 3.114 (±1.2), with a maximum number of six nucleotide differences. Phylogenetic analysis of these 31 haplotypes supported the monophyly of *C. elephas* when *C. glandium* was used as an outgroup (data not shown here). The majority of haplotypes occurred as an unresolved cluster and the remaining haplotypes were distributed among three major clades with substantial structure. These major clades (clade 1, 2 and 3) were supported with high (>0.85) posterior probability values (Fig. 1b). Even though clade 1 consisted of haplotypes that were found only in population "Melivia" (4) the other two clades showed substantial structure. Clade 2, contained two subclades (2-1 and 2-2) with geographically isolated haplotypes (haplotypes of subclade 2-1 were found only in population "Aghiasos" (5) and the ones of subclade 2-2 only in population "Halkida"(6)), whereas the other two haplotypes (Ele 19 and Ele 28) could be found in populations "Arnea" (10), "Halkida" (6), and "Western Crete" (7). Clade 3 had only one statistically supported deeper subclade (3-1) that was present in the populations "Paiko" (9), "Kastri" (8), and "Aghio Oros"(1) and one more haplotype (Ele 16) that was spread in populations "Aghio Oros" (1), "Western Crete" (7), and "Eastern Crete" (2).

The parsimony network did not contain any reticulations, indicating the absence of homoplasy among haplotypes. Evaluation of the total network (Fig. 1c) as inferred from GeoDis revealed that the current pattern of differentiation resulted from "restricted gene flow with isolation by distance.". The pattern of isolation by distance was also tested using a Mantel test that rejected the null hypothesis of no association between genetic (Tamura–Nei) and geographic distances (*g* = 3.2357, *p* = 0.01). Analysis of molecular variance (AMOVA), however, did not show a strong genetic structure when populations were grouped according to various geographic combinations (data not shown) whereas SAMOVA showed that the value of ΦCT was larger when *K* = 2 indicating that partitioning into two groups was more appropriate. Several different approaches, including mismatch analysis (Fig. 2b), LTT (Fig. 2a) and Tajima's *D* statistic (*D* = −2.25371 and *p* < 0.01) (Table 2), indicated a consistent population expansion of *C. elephas*.

Dating the divergence times between the major phylogenetic clades, suggested that clades began to diverge after the "Mindel" ice age that ended about 300.000 before present (BP) in the Alpine regions. By the start of the following ice age (Riss), the major clades of *C. elephas* in Greece began formed (Table 3). The average migration rate estimate was *M* = 0.173, suggesting that on average one female was exchanged between the clades about every six generations. However, this rate was significantly lower (*M* = 0.14) among the well-defined clades compared to the

Table 2 Haplotype diversity (h), nucleotide diversity (π), and Tajima's D statistics calculated from the 790 bp mitochondrial DNA locus of *C. elephas*

No. samples	No. haplo types	Haplotype diversity (h)	Nucleotide diversity (π)	Tajima's D
160	31	0.7041 ($\pm$0.0405)	0.001668 ($\pm$0.001157)	-2.25371 ($p < 0.01$)

Fig. 2 a Lineage through time plot and **b** mismatch distribution diagram inferred from the mitochondrial DNA sequences of *C. elephas* in Greece

Table 3 Divergence times in years before present (below diagonal) and M (migration) rates (above diagonal) between the major phylogenetic clades estimated by MDIV

	Clade 1	Clade 2	Clade 3	Remaining HT
Clade 1		0.14	0.14	0.22
Clade 2	223,036		0.14	0.18
Clade 3	204,892	199,722		0.22
Remaining HT	214,442	292,125	279,110	

and the Mantel tests support this observation and suggest that restricted gene flow with isolation by distance shaped the current pattern of intraspecific diversity of *C. elephas*.

Pleistocene environment likely influenced the genetic diversity of *C. elephas* in Greece. During Pleistocene, Greece experienced repeated periods of extreme cold with a roughly 100,000 years cycle interrupted by short warm interglacial periods (Messerli 1967), similar to the ones known in the Alps and northern Europe. The glacial sequences, Skamnellian, Vlasian, and Tymphian stages, in Greece (Sibrava et al. 1986; Tzedakis et al. 1997) have the equivalent names Mindel, Riss, and Würm respectively, which apply to the classic Alpine morphostratigraphical periods (Penck and Brückner 1909). Our results suggest that the main divergence events began during the Skamnellian stage and emerged in the subsequent interglacial stage (300000–190,000 years before present), before the Vlasian stage began. The origin of divergence in the Skamnellian stage seems plausible as this glacial stage gave rise to the most extensive glacial deposits (Hughes et al. 2003; Woodward et al. 2004) which separated the populations of *C. elephas* and initiated lineage divergence. The glaciers formed during these ice ages, and particularly during the Vlasian and Tymphian stages were of a lesser magnitude (Hughes et al. 2003) and likely allowed for movement of weevils among regions and subsequent gene flow.

Contemporary factors may have also influenced the observed distribution of haplotype diversity. A rough estimation of migration rates among the different clades indicates an average migration of one female every six generations. In the case of chestnut weevil, gene flow may have been increased due to human activities, due to the historical cultivation of chestnut (Conedera et al. 2004). Chestnuts have been commonly transported all over Greece and consequently, weevils were also transported to distant

rates estimated between the smaller clades and the cluster of remaining haplotypes ($M = 0.18$–0.22) (Table 3).

Discussion

Our results suggest that divergence among the clades of *C. elephas* in Greece occurred during the Pleistocene approximately 200,000 years before present (Table 3). The broad distribution pattern of *C. elephas* in Greece, in concert with their recent phylogenetic divergence (only six nucleotides differed among the 790 mitochondrial DNA nucleotides) suggest that isolation by distance contributed to the occurrence of unique haplotypes/clades in populations peripheral to "Hortiatis" (3), "Melivia" (4), and "Arnea" (10). The outcome of the Nested Clade analysis

regions. The human-mediated transport of chestnut weevils has quite likely blurred the phylogeographic signal of *C. elephas* in Greece resulting in the current distribution of genetic diversity. A comparison between the haplotype diversity for *C. elephas* with that of other *Curculio* species supports further the action of a homogenizing factor among pest's populations in Greece. Aoki et al. (2008) found 114 haplotypes among 204 *C. hilgendorfi* samples while Aoki et al. (2009) identified 41 haplotypes among the 115 *C. sikkimensis* individuals analyzed. Only Toju and Sota (2006) found comparable results, with 23 haplotypes among the 246 individuals of *C. camelliae* analyzed. Gene flow was suggested as a plausible explanation for the observed diversity (Toju and Sota 2006). Gene flow should have affected the observed distribution of haplotypes of *C. elephas* in Greece.

Curculio elephas populations are expanding (Table 2). This pest exhibits the potential to proliferate rapidly which underlines the urgent need for a strategy to restrict its movement. Chemical insecticides are commonly used to control *C. elephas* in Greece. The increased use of these chemicals throughout the potential range of *C. elephas* is not recommended because of their environmental impact. Species-specific biological agents for the control of *C. elephas* would minimize environmental impact. Entomopathogenic fungus and nematodes are potentially efficient control agents of chestnut weevil (Paparati and Speranza 1999; Kepenekci et al. 2004; Karagoz et al. 2009). The effectiveness of these entomopathogens was deduced from laboratory tests and limited field trials thus generalization of their effectiveness throughout the potential range of *C. elephas* is limited. Field experiments with entomopathogenic nematodes, fungus and other biological control agents are needed to test the efficiency of these agents throughout the potential range of *C. elephas*.

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