Clustered Microsatellite Mutations in the Pipefish *Syngnathus typhle*

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ABSTRACT

Clustered mutations are copies of a mutant allele that enter a population's gene pool together due to replication from a premeiotic germline mutation and distribution to multiple successful gametes of an individual. Although the phenomenon has been studied in Drosophila and noted in a few other species, the topic has received scant attention despite claims of being of major importance to population genetics theory. Here we capitalize upon the reproductive biology of male-pregnant pipefishes to document the occurrence of clustered microsatellite mutations and to estimate their rates and patterns from family data. Among a total of 3195 embryos genetically screened from 110 families, 40% of the 35 detected *de novo* mutant alleles resided in documented mutational clusters. Most of the microsatellite mutations appeared to involve small-integer changes in repeat copy number, and they arose in approximately equal frequency in paternal and maternal germlines. These findings extend observations on clustered mutations to another organismal group and motivate a broader critique of the mutation cluster phenomenon. They also carry implications for the evolution of microsatellites with respect to mutational models and homoplasy among alleles.

A genetic alteration that arises premeiotically in the germline can be distributed to multiple gametes (Figure 1a). Thus, copies of the same new mutation may enter a population's gene pool simultaneously in a "cluster" (Woodruff and Thompson 1992; Huai and Woodruff 1997). Such clusters of new mutants contradict a common assumption of many population genetics models: that mutations occur independently and enter the gene pool as singletons (as might apply, for example, to mutations arising late in meiosis or during gametic maturation; Figure 1b). Woodruff *et al.* (1996) argue that an appreciation of the clustered mutation phenomenon "may cause us to reconsider many of the fundamental relationships on which population genetics theory is based" (p. 149).

Empirical data from Drosophila suggest that mutational clusters may account for 20–50% of all new mutant alleles entering a population (Woodruff and Thompson 1992; Woodruff *et al.* 1996). However, little attention has been directed toward the phenomenon in other species, beyond occasional reports of genotypic clusters primarily in humans and some other mammals (*e.g.*, Schreider 1969; Vogel and Rathenberg 1975; Russell *et al.* 1981; Edwards 1989; Ehling and Newhauser-Klaus 1989; Woodruff and Thompson 1992). Here we estimate, from family data in fishes, rates and patterns of microsatellite mutations. Results are relevant to the mutation-cluster scenario as well as to the nature and implications of molecular changes at microsatellite loci.

This article is a byproduct of genetic analyses of parentage and mating systems in fishes of the pipefish and seahorse family Syngnathidae (Jones and Avise 1997a,b; Jones *et al.* 1998), all members of which exhibit male pregnancy (Vincent *et al.* 1992). In *Syngnathus typhle*, one or more females deposit eggs through a small opening into a male's brood pouch, where he fertilizes them and houses the resulting embryos for several weeks until parturition (Berglund *et al.* 1986a,b, 1988). Internal fertilization means that for the brooding male paternity is assured for all offspring within his pouch (Jones and Avise 1997b). In genetic analyses, assurance of paternity facilitates maternity assignments, which in turn permit the identification of *de novo* mutations in brooded embryos and assignment of their place of origin to the paternal or maternal germlines.

MATERIALS AND METHODS

Samples and microsatellite procedures: The characterization of *S. typhle* microsatellites followed standard techniques (*e.g.*, Jones *et al.* 1998) and is reported elsewhere (Jones *et al.* 1999), as are polymerase chain reaction (PCR) conditions and primer sequences. A total of four microsatellite loci were used for the current study. Mutations were observed for the two tetranucleotide loci, *typh*04 and *typh*16, while the two dinucleotide loci (*typh*12 and *typh*18) were used to provide additional resolution of parentage in initially ambiguous cases.

Fin clips of frozen adults and individual brooded embryos were employed as tissue sources. During dissection of pregnant males, the spatial position of each embryo within a brood

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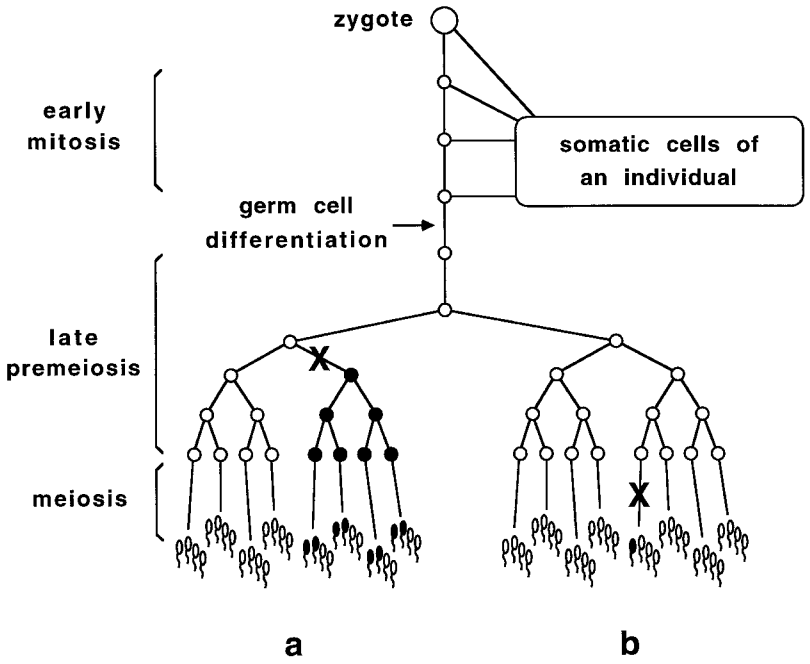

Figure 1.—Schematic of cell lineages during ontogeny (modified from diagrams in Woodruff and Thompson 1992; Woodruff *et al.* 1996). Mutations are denoted by X. (a) A mutation arises premeiotically and is replicated into a cluster of copies in multiple descendant gametes; (b) another mutation arises in late meiosis in a single gametic cell.

pouch was mapped. DNA was prepared for PCR using techniques described in Jones and Avise (1997a). Precise assay conditions in the current study are described elsewhere (Jones *et al.* 1999).

Genetic data were obtained from broods of 110 males collected from Gullmar Fjord, Sweden, an area with no major pollution or industrial outlets. Thirty of the males (labeled FCST) were pregnant when caught. The remaining 80 were not pregnant at time of capture but subsequently were used in controlled laboratory experiments involving crosses with known females.

Identification and assignment of new mutations: Single-locus genotypes of each pregnant male were compared against those of each embryo within his brood pouch. Normally, each of the two alleles from the father appeared in about one-half of his offspring, in accord with Mendelian expectations. However, in a small fraction of cases, one or more embryos displayed (invariably at one locus only) a heterozygous genotype with neither allele identical-in-state to the two alleles in the known sire. These unusual alleles presumably resulted from mutations that arose in the paternal germline.

By subtraction of the paternally derived allele from each embryo's diploid genotype, the allele contributed by the biological mother could be deduced. For the 80 families resulting from laboratory crosses, maternity of embryos was assigned unambiguously from a small pool of potential mothers (two to six) using from two to four microsatellite loci. Usually, at a given locus, both alleles from a mother segregated in her offspring as expected. However, in a small fraction of cases, one or more embryos displayed a heterozygous genotype with neither allele identical-in-state to those of the mother (who was identified unambiguously on the basis of data from up to three additional loci). These embryos carried mutations that presumably originated in the maternal germline. For most families, an allele identical-in-state to the putative mutant allele was not present in any of the potential mothers in the breeding tank, providing convincing evidence of maternally derived mutations.

A potential complication arises in identifying maternal-origin mutations in the broods of the 30 males that were already pregnant when collected. If more than one female deposited eggs in a male's brood pouch, what might appear to be a new mutation from the maternal line could represent an embryo from a different mother. To safeguard against such errors of assignment, procedures described in Jones and Avise (1997b) were used to identify instances of multiple maternity and to reconstruct maternal genotypes from joint multilocus genotypic examinations of embryos. A mutant allele was inferred only in those cases in which (1) the embryo's genotype could not be explained without invoking the presence of an additional mother for that single embryo; and (2) the embryo appeared to be the progeny of one of the other inferred females of the brood based on data from the three additional loci. In the current study, a male's brood did indeed often contain embryos of multiple females. Invariably, as was true in earlier genetic analyses of a related species, *S. floridae* (Jones and Avise 1997b), multiple embryos from different mothers were spatially grouped within a male's brood pouch. Thus, distinct maternal cohorts of embryos within each brood pouch were evident, and the identification of new mutations from the maternal side was not compromised severely.

RESULTS

The four loci used in this study were highly polymorphic (15–39 alleles per locus), facilitating the reconstruction of maternal genotypes, the assignment of parentage in breeding experiments, and the identification of novel size variants (Jones *et al.* 1999). In particular, *typh*04 and *typh*16 displayed 39 and 20 alleles, respectively, in samples of 293 adults.

Twenty-six independent *de novo* mutations were detected in this study—20 at locus *typh*04 and 6 at *typh*16. Their various features are described in Table 1. Fourteen of the independent mutations arose from progenitor alleles on the paternal side and 11 on the maternal side. One mutation was ambiguous with respect to paternal *vs.* maternal origin, because both parents carried

TABLE 1

Description of the 26 independent *de novo* mutations observed in a microsatellite survey of *Syngnathus typhle*

Male ID	Cluster size[b]	No. progeny assayed	Genotype			Mutation[a]	Germline origin
			Father	Mother	Embryo		
Single mutations at *typh*04							
MT21-2	1	70	197/213	175/233	213/229	233→229	Maternal
MT22-1	1	25	205/205	167/205	167/209	205→209	Paternal
MT22-2	1	3	217/229	209/233	217/229	233→229	Maternal
MT26-1[c]	1	10	205/237	201/237	237/241	237→241	Undetermined
MB2-2	1	31	185/213	213/269	189/269	185→189	Paternal
MB-82	1	4	189/237	205/221	209/237	205→209	Maternal
MT1-2	1	23	213/229	171/217	209/217	213→209	Paternal
MT1-3	1	47	175/241	193/201	175/197	193[d]→197	Maternal
FCST2	1	23	191/209	197/null	197/217	209→217	Paternal
FCST6	1	60	213/221	205/217	217/217	213→217[e]	Paternal
FCST8	1	30	171/201	225/237	201/233	237→233	Maternal
FCST11	1	42	197/245	201/205	201/237	245→237	Paternal
FCST12	1	61	205/225	189/217	189/229	225→229	Paternal
FCST20	1	42	189/229	171/229	185/229	189→185	Paternal
FCST27	1	15	193/213	179/221	213/225	221→225	Maternal
FCST34	1	5	183/185	193/225	185/213	225→213	Maternal
Clustered mutations at *typh*04							
MT11-1	5	13	189/213	197/209	197/217	213→217	Paternal
					209/217	213→217	Paternal
				185/229	185/217	213→217	Paternal
					185/217	213→217	Paternal
					217/229	213→217	Paternal
MT19-1	2	33	209/233	213/217	213/229	233→229	Paternal
					217/229	233→229	Paternal
FCST20	3	42	189/229	171/229	171/225	229→225	Paternal
					171/225	229→225	Paternal
					171/225	229→225	Paternal
FCST26	2	59	185/217	191/205	189/205	185→189	Paternal
					189/205	185→189	Paternal
Single Mutations at *typh*16							
MT3-2	1	23	235/267	247/275	271/275	267→271	Paternal
MB6-4	1	18	263/267	235/255	259/267	255→259	Maternal
MT29-1	1	15	259/271	235/251	239/259	235→239	Maternal
FCST2	1	7	225/263	251/271	247/263	251→247	Maternal
FCST55	1	50	247/307	259/263	263/303	307→303	Paternal
Clustered mutations at *typh*16							
MT29-1	2	9	259/271	235/235	231/271	235→231	Maternal
					231/271	235→231	Maternal

[a] Lengths of the ancestral and derived (mutant) sequences, in base pairs.
[b] Observed numbers of offspring carrying a copy of the same mutant allele.
[c] Which parent produced the mutant gamete could not be determined in this case, because both the father and mother carried the 237 allele.
[d] Alternatively, the original allelic state could have been 201, but in either case only one 4-bp change was involved.
[e] Alternatively, the original allelic state could have been 221, but in either case only one 4-bp change was involved.

the allele 237 and the embryo displayed the genotype 237/241.

The original cloned sequences for *typh*04 and *typh*16 contained, respectively, the microsatellite regions $(GGTT)_5(GGAT)_6$, corresponding to allele size 191, and $(GATG)_{15}$, allele 239. All of the mutations observed at these tetranucleotide repeat loci involved insertions or deletions of 4, 8, or 12 nucleotides, suggesting the addi-

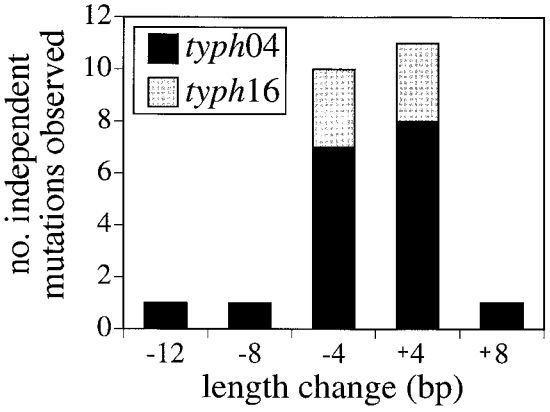

Figure 2.—Histogram of sequence-length changes characterizing the 26 *de novo* mutations observed in *S. typhle* at two microsatellite loci with tetranucleotide repeat motifs.

tion or removal of one to three tetranucleotide-motif units. Figure 2 shows a compilation of mutation sizes under the assumption that each mutant allele arose from its closest-sized counterpart in the relevant parental line. Twenty-three of the mutations (88%) differed from their respective progenitors by a single repeat unit (4 bp), 2 (8%) differed by two repeat units, and 1 (4%) differed by three repeat units.

Within each of five broods, multiple copies of a mutation were observed (Figure 3). For example, 3 among the 42 embryos assayed from pregnant male FCST20 (Table 1) displayed the same-state mutant allele, deduced in this case to be of paternal origin. Such cases evidence the clustered-mutation phenomenon wherein, presumably, a single mutation that arose in the germline was replicated and distributed to multiple gametes that eventuated in the embryos assayed. Four of these mutational clusters originated paternally and one arose on the maternal side.

One additional family (not listed in Table 1) that displayed clustered copies of a mutation warrants special mention. This involved a laboratory cross between a male (MB6-1) and a known female who also was genotyped directly. Among the 21 embryos scored at *typh*04 were the two expected paternal alleles (196 and 201) in similar frequencies, but also three alleles (209, 213, and 225) deduced to be of maternal origin. Interestingly, all three maternal-origin alleles also were present in the gel profile of the mother (Figure 4), which represents DNA amplified from the somatic cells of her caudal fin. This result was confirmed in multiple embryos as well as multiple independent tissue samples from the adult female. The mother displayed a normal, Mendelian-segregating genotype at *typh*16 (Figure 4). This pattern most likely resulted from germline and somatic mosaicism at *typh*04 in this female (see below).

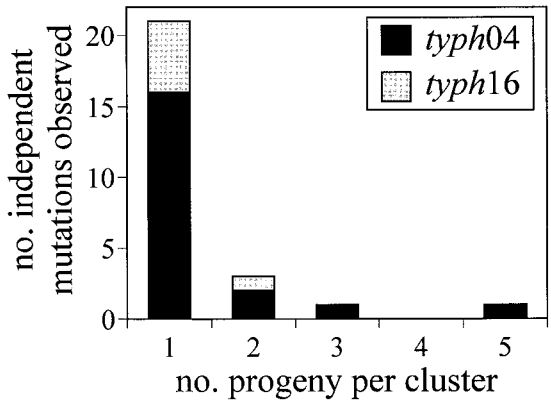

Figure 3.—Histogram showing the number of copies per cluster observed for the 26 independent *de novo* mutations recorded in *S. typhle* at two microsatellite loci.

DISCUSSION

Molecular features of microsatellite mutations: Most of the independent mutations observed in *S. typhle* appear to have altered the length of the microsatellite sequence by a single repeat unit (4 bp), and the remainder changed the repeat copy count by only 2 or 3 units (Figure 2). These alterations are considerably smaller than the total span of allelic sizes observed in the population sample: at *typh*04, alleles ranged in length from 165 bp to 269 bp (a span of 26 repeat units), and at *typh*16 from 223 bp to 307 bp (21 repeat units). These findings are consistent with suspected tendencies for most microsatellite loci to mutate stepwise rather than by saltatory changes in repeat count (Dallas 1992; Shriver *et al.* 1993; Valdes *et al.* 1993; Weber and Wong 1993; Di Rienzo *et al.* 1994; Bruford *et al.* 1996; Jin *et al.* 1996; Primmer *et al.* 1996).

A potential circularity in this conclusion stems from the fact that each new allele was assumed provisionally to have arisen from its size-closest counterpart in the relevant parental genotype. However, a scenario in which saltatory changes resulted in the observed pattern of mutations is difficult to imagine. For example, if we assume that some or all of the mutations had arisen from the most distant allele in the parent (Table 1), then on every occasion the mutation event produced a new allele that was very close in size to the other, unchanged parental allele. We might envision mechanisms involving gene conversion or interallelic recombination resulting in such a pattern, but the simplest explanation of our data is that the mutations seen here involved changes of one or a few repeat units.

All except one of the observed mutations resulted in alleles whose sizes were identical to those of alleles already present in the population. These results support other studies suggesting that size homoplasy at microsatellite loci is a widespread phenomenon (Estoup *et al.* 1995; Angers and Bernatchez 1997; Orti *et al.* 1997).

Clustered mutations: Woodruff and colleagues

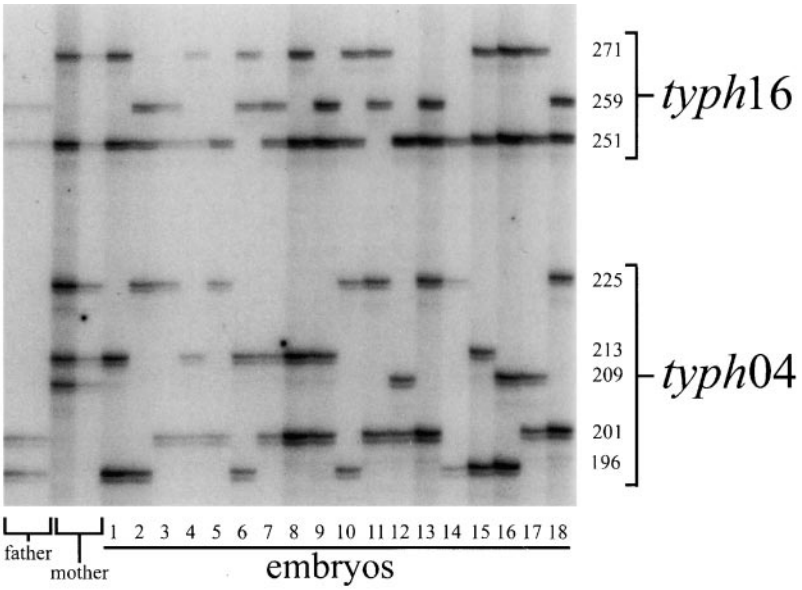

Figure 4.—Autoradiogram of the gel showing the germline and somatic mosaicism evident in one female assayed as part of a controlled laboratory cross. The loci *typh*16 and *typh*04 were multiplexed and resolved on a single gel. The 18 numbered lanes to the right are the genotypes of the embryos produced from a cross between male MB6-1 and female F118-2. The leftmost two lanes represent MB6-1's genotype, which segregated in progeny according to Mendelian expectations at both loci. The next two lanes represent the genotype of F118-2 (assayed independently from two separate tissue samples). For *typh*16, this female's gametes satisfied Mendelian expectations, but for *typh*04, F118-2 displayed a three-banded pattern (209/213/225), and all three alleles were passed to progeny. The most likely explanation is that F118-2 underwent a mutation early in development before the differentitation of the germline and soma and that this led to somatic mosaicism and clustered mutations in her progeny (see text).

(1996) have argued persuasively that clustered mutations have been overlooked in population genetics and that proper consideration of their ramifications "may alter our views of the evolutionary landscape" (p. 149). The current data permit commentary on various aspects of this suggestion from an empirical basis.

One immediate consequence of clustered mutations is that identical variants in effect arise at once and are distributed to multiple siblings, resulting in a patchy temporal and spatial arrangement within a population. In this collection of pipefish, several instances of clustered mutations were identified by the appearance of identical-state copies of a mutant allele in multiple embryos within a brood. Five embryos were observed in the largest of these mutational clusters, but this and other such numbers represent minimum estimates of cluster size because broods may have contained additional copies of the mutation in embryos not assayed. In nature, such clustered mutations among sibling pipefish would indeed begin existence in the population in discrete spatial and temporal groups.

Depending upon accounting procedures, another consequence of clustered mutations is that they can impact interpretations of mutation numbers and rates. For example, when the multiple copies within each mutational cluster are included in the tally, the total number of newly appearing mutations in this pipefish generation increases by 35%, from 26 to 35. Another way of looking at this is to note that among the 35 new mutant alleles observed in the embryo generation, 14 (40%) occurred in mutational clusters. The observed fraction of newly arisen pipefish alleles residing in mutational clusters is similar to reported estimates (20–50%) in Drosophila (Woodruff and Thompson 1992; Woodruff *et al.* 1996).

With respect to mutation rate, 26 *independent* mutations and 35 total new alleles were detected among the total of 3195 pipefish embryos assayed at two loci each in this study. The former value yields an estimated mutation rate of $\mu = 2.0 \times 10^{-3}$ (3.1×10^{-3} for *typh*04 and 9.4×10^{-4} for *typh*16), whereas the latter yields an estimate 35% higher: $\mu^* = 2.7 \times 10^{-3}$. These values are within the range reported for microsatellite loci in other species (Weber and Wong 1993; Primmer *et al.* 1996; Schug *et al.* 1997). Both tallying schemes are valid but provide somewhat different information. The estimate μ should be interpreted as the rate of mechanistic mutational occurrence, whereas μ^* represents the appearance rate (or frequency) of new alleles entering a population per generation.

In general, these two estimates will differ depending upon (among other factors) the origination time of particular germline mutations during ontogeny. A neutral mutation that arises extremely early in the differentiation of the germline (Figure 1) will be distributed to nearly 50% of an individual's gametes, whereas a late-meiotic origin for that same mutation could result in its confinement to a single sex cell. If a mutation arose before differentiation of the germline and the soma, a pattern like that observed in the progeny of male MB6-1 (Figure 4) might arise. In this case, a mutation that arose early in the development of the mother of the embryos could have resulted in both germline and somatic mosaicism for the mutant allele in this female. Such a pattern, provisionally explained by an early mutation resulting in mosaicism, has been observed in other vertebrates (Kelly *et al.* 1991; Bullman and Whittaker 1994; Zlotogora 1998). However, other explanations may be possible, such as early fusion of two embryos or zygotes (*e.g.*, Green *et al.* 1994; Giltay *et al.* 1998), triploidy, or trisomy. Our results do not conclusively rule out any of these possiblities.

In terms of population genetic effects, the two ontogenetic extremes of mutation origin (early *vs.* late during germline formation) and, hence, the two procedures of mutational accounting, can be viewed as equivalent but one generation out-of-step to one another. Thus, a mutation counted as a single event early in a diploid individual's life in a parental generation of size N (*i.e.*, mutation frequency $= 1/2N$) alternatively could be tallied as the frequency of that mutant allele in the next generation (*i.e.*, $X/2NX = 1/2N$, assuming that each individual leaves X offspring). As long as this accounting phenomenon is recognized, the appearance of mutations in clusters has little effect on most aspects of population genetics theory. On the other hand, under certain restricted cases clustered mutations may have some population-level consequences, for example when mutations are under- or overdominant (Woodruff *et al.* 1996) or when clustered mutations are especially prone to union into homozygous zygotes via local population inbreeding (Woodruff and Thompson 1992).

The screening procedure for mutational clusters in pipefish was facilitated by the known genetic paternity (and inferred maternity) of relatively large broods of offspring. In other regards, the current setting was less than ideal for describing frequencies and distributions of mutational clusters. In comparable-intent studies of Drosophila, for example, balancer stocks and controlled crosses were used to extract and then examine nearly 2 million chromosomes for mutations with readily scorable phenotypic effects (Mason *et al.* 1985; Woodruff and Thompson 1992). The fact that we were able to identify clustered mutations in pipefish using modest sample sizes was made possible by the unusually high mutation rates at microsatellite loci. Additional studies of this kind will extend an understanding of the mutation-cluster phenomenon to other taxa and to other genetic systems.

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