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## New contact zone and assemblages among water frogs of Southern France

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### Abstract

Water frogs are involved in several hybridogenetic complexes. However, investigations in Southern and Western France produced evidence of new hybridization events between *R. lessonae* and *R. perezi* (some new hybrids tadpoles were discovered), and the existence of new assemblages [R–G, L–P, R–P, P–R–G: (*R. ridibunda*; *G. grafi*; *L. lessonae*; *P. perezi*)]. In respect to hybridogenesis, these assemblages offer opportunities for both primary and secondary hybridizations. Such peculiarities may be explained either by introductions or by relic populations and may influence water frog evolution.

**Key words:** *Rana ridibunda* – *R. kl. esculenta* – *R. lessonae* – *R. perezi* – *R. kl. grafi* – hybrid zone – hybridogenesis – LP tadpoles

### Introduction

Hybrid zones are known to be places where events of evolutionary importance such as reinforcement, genetic reassortment, polyploidization, initiation of clonal (or hemiclinal) hybrid lineages may occur. These mechanisms may in turn promote differentiation and/or speciation events (Bullini 1994; Ptacek et al. 1994; Vrijenhoek 1994; Arnold 1997). Among different processes of genetic reassortment, examples of introgressive hybridization are so common even in animals (e.g. DeMarais et al. 1992; Echelle and Echelle 1994; Katoh and Ribi 1996; Sumida and Ishihara 1997; Greg et al. 1998) that Dowling and DeMarais (1993) argued for the evolutionary importance of introgressive hybridization.

European water frogs form peculiar hybrid zones because they may combine asexual and sexual reproduction (Schmidt 1993). Several species in this group can give rise to hybrid lineages exhibiting a hybridogenetic reproductive mode characterized by genome exclusion in the hybrid germ line and by the production of clonal gametes which only contain the genome from one of the parental species (Schultz 1969; Tunner and Heppich-Tunner 1991). There is usually no recombination between the parental genomes, but introgression has been documented for both the mitochondrial (Plötner 1998) and the nuclear genome (review in Pagano and Schmeller 1999). As introgressive hybridization may often be a consequence of introduction of non-native species (Echelle and Echelle 1994; Greg et al. 1998), and as the presence of non-native water frogs has been shown (Pagano et al. 1997), a first step in studying water frog hybrid zones is to infer the impact of exotic introductions on the composition and reproduction of native populations.

Water frogs can be found to form several hybridogenetic complexes (review in Graf and Polls-Pelaz 1989). The most widespread complex occurs in Central and Eastern Europe (Graf and Polls-Pelaz 1989). In this complex, the hybridogen *Rana kl. esculenta* (genome RL) originated from 'primary' hybridization between *Rana ridibunda* (genome RR) and *Rana lessonae* (genome LL). The hybridogens usually coexist only with the sexual parental host species *R. lessonae*. Matings between the hybridogen *R. kl. esculenta* and *R. lessonae* (as 'secondary' hybridizations) maintain hybridogenetic lineages as *R. kl. esculenta* transmits only its *ridibunda* genome to haploid

gametes (L–E system, Uzzell and Berger 1975). Such host–hybridogen populations constitute persisting reproductive systems. However, in natural populations, other taxa may coexist without forming hybridogenetic systems. Pagano et al. (1997) termed these coexisting taxa 'assemblages' in contrast to the 'systems' which represent host–hybridogen persisting populations. Parental species or hybridogens can also constitute 'pure' populations (Günther et al. 1979; Graf and Polls-Pelaz 1989).

Another hybridogenetic complex occurs in Southern France and Northern Spain (e.g. Graf et al. 1977; Graf and Polls-Pelaz 1989; Pagano et al. 2001). In this complex, the origin of the hybridogen (*R. kl. grafi*, genome RP) remains speculative because hybridogens may originate from a 'primary' hybridization between *Rana perezi* (genome PP) and either *R. ridibunda* (e.g. Graf et al. 1977), or *R. kl. esculenta* (e.g. Arano et al. 1995a). In the P–G system (analogous to the L–E system), hybridogenetic lineages are restored at each generation through matings of the hybridogen *R. kl. grafi* with *R. perezi*, as *R. kl. grafi* transmits only its *ridibunda* genome to haploid gametes (Graf and Polls-Pelaz 1989).

It has been predicted (Graf and Polls-Pelaz 1989) that the *grafi* complex (*R. perezi*, *R. kl. grafi*) and the *esculenta* complex (*R. ridibunda*, *R. lessonae*, *R. kl. esculenta*) may be in contact in Southern France. There, different assemblages may occur between the sympatric taxa and may offer opportunity for other hybridization events. In addition to these two complexes, other water frog species (such as *Rana bedriagae*, *Rana shqipericana*, *Rana kurtmuelleri*) or non-native *R. ridibunda* frogs (mainly from Balkan countries) have probably been introduced into natural populations through frog importation (Arano et al. 1995b; Pagano et al. 1997; Pagano et al. 2001). Such introductions may affect water frog reproduction (i) because they modify taxa distribution and so potentially offer opportunities for exotic frogs to coexist and mate with native taxa (constituting new assemblages); and (ii) because Balkan *R. ridibunda* are resistant to hybridogenesis (Hotz and Uzzell 1983; Guerrini et al. 1997) and may produce either hybridogenetic systems or hybrid complexes (not necessarily hybridogenetic) in which introgression may occur.

With this in mind, the aims of our study were to investigate water frog populations in Southern France in order to determine their taxonomic composition. Specifically, we (i)

established the sympatric occurrence of *R. lessonae* and *R. perezi*; (ii) determined to what extent taxon composition conformed to pure populations and persisting hybridogenetic systems or to 'assemblages'; and (iii) identified new assemblages and new hybridization events that may have influenced water frogs evolution.

## Material and methods

Most sampling was performed at night during the breeding season (in 1995–98). In addition, some samples were caught by day away from the breeding habitats. A total of 583 adults frogs were sampled in 36 different localities (Fig. 1; see Pagano et al., 2001 for details on localization). The relative taxonomic composition of each pond was specified by an allozymic identification of each frog using five allozymic specific markers: lactate dehydrogenase (LDH-B; E.C. 1.1.1.27), glycerol-3-phosphate dehydrogenase ( $\alpha$ GDH; E.C. 1.1.1.8), s-adenosyl-l-homocysteine hydrolase (AHH; E.C. 3.3.1.1), mannose-phosphate-isomerase (MPI, E.C. 5.3.1.8), phosphoglucomutase (PGM-2, E.C. 2.7.5.1).

Methods for tissue homogenization, composition of buffer and starch gels, migration and staining solutions were published in previous studies (see, e.g. Pagano et al. 1997).

At the Bayonne pond, six frogs in amplexus were caught and identified. In addition, tadpoles were sampled, identified by allozymic markers and reared.

Catching frogs at night during the breeding season introduces a bias in sampling (males are more frequently caught than females because they form choruses and call). We, however, used this method because of its better sampling efficiency.

## Results

In seven ponds (19% of the ponds) we found several assemblages and a pure hybrid population (Table 1) previously undescribed: (1) *R. ridibunda* + *R. perezi*; R–P assemblage; (2) *R. ridibunda* + *R. kl. grafi*; R–G assemblage; (3) *R. ridibunda* + *R. perezi* + *R. kl. grafi*; P–R–G assemblage; (4) *R. lessonae* + *R. perezi*; L–P assemblage; and (5) a putative pure *R. kl. grafi* population (although the sample sizes do not permit us to conclude definitively to the existence of a pure hybridogen population).

These new assemblages are located in southern and western France in sites separated by distances of hundreds of kilometres and are distributed across the country either along the Atlantic coastal regions or in Mediterranean areas (Fig. 1).

Table 1. Occurrence of different populations and assemblages discovered

	No. of populations	No. individuals in each population
P–R–G assemblage	1	18
R–P assemblage	1	2
G population	1	7
R–G assemblage	3	3; 7 and 9
L–P assemblage	1	6

The population composition and sex composition of these newly described populations or assemblages are indicated in Table 2. Although sex ratio is biased, our data suggested that males of different taxa may compete for females in the P–R–G assemblage at Salins de Badons, in the R–P assemblage at Les Sables d'Olonne, and in the three R–G assemblages. In some sites where females were sampled, females have the choice between males of two or three different taxa (in the P–G assemblage of St Mathieu de Trévières and in the P–R–G assemblage of Salins de Badons, respectively). In contrast with this general male excess, only females were found in the putative pure *R. kl. grafi* population.

Our data provide evidence for natural hybridization events between *R. lessonae* and *R. perezi*. At the Bayonne pond, we found some unexpected matings between *R. lessonae* males and *R. perezi* females. In the same pond, we also identified 19 LP hybrid tadpoles containing both a *perezi* and a *lessonae* genome, demonstrating that hybridization indeed happened (Fig. 2 and Table 3). This is the first documented occurrence of hybridization between *R. lessonae* and *R. perezi* in the wild.

In 29 ponds, we found 531 frogs (91% of our total sampling) constituting either pure populations or assemblages or hybridogenetic systems that have been previously observed (Graf and Polls-Pelaz 1989; Pagano et al. 1997). We found 'pure' *R. ridibunda* populations (in seven ponds), 'pure' *R. perezi* populations (in four ponds), 'pure' *R. lessonae* populations (in two ponds), 'pure' *R. kl. esculenta* populations (in three ponds), R–E assemblages (in two ponds), L–E–R assemblage (in one pond), L–E systems (in six ponds), P–G systems (in four ponds).

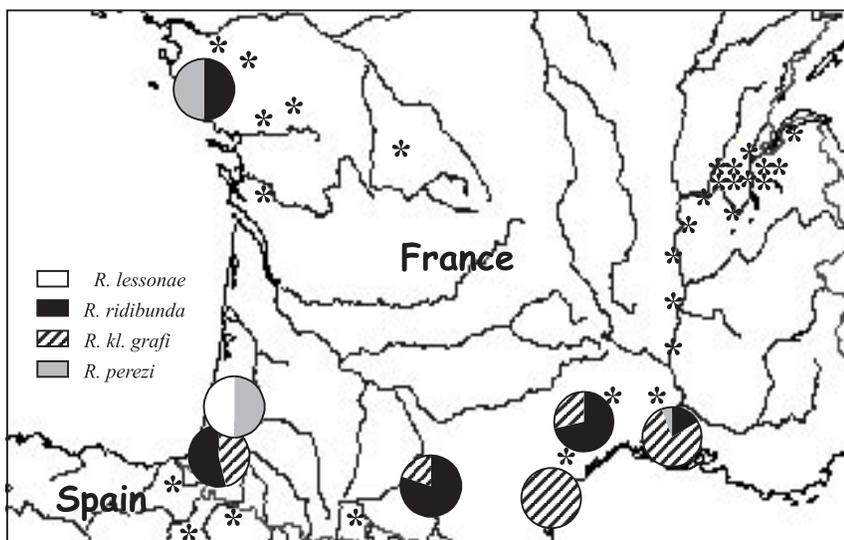


Fig. 1. Location of sites where frogs were sampled and identified. Stars represent sites where populations, systems or assemblages previously published where recorded. The seven sites where new assemblages were discovered are represented by the circles which specify the relative percentage of each taxa in each pond

Table 2. Population composition and observed sex-ratio in the new assemblages discovered

Site (assemblage)	<i>R. perezii</i>	<i>R. ridibunda</i>	<i>R. kl. grafi</i>	<i>R. lessonae</i>	Habitat type
Salins de Badons (P-R-G assemblage)	1M (+ 1J)	2M (+ 1J)	3F + 4M (+ 6J)	-	Marsh
Pissevache (G 'pure' population)	-	-	6F (+ 1J)	-	Marsh
Orx (R-G assemblage)	-	4M	5M	-	Marsh
Toulouse (R-G assemblage)	-	2M	1M	-	Shallow pond
Bayonne (L-P assemblage)	3F	-	-	3M	Shallow pond
Sables d'Olonne (R-P assemblage)	1M	1M	-	-	Large pond
St Mathieu Tréviers (R-G assemblage)	-	2M + 4F	1F	-	Gravel pit

J means juvenile; F, female; M, male.

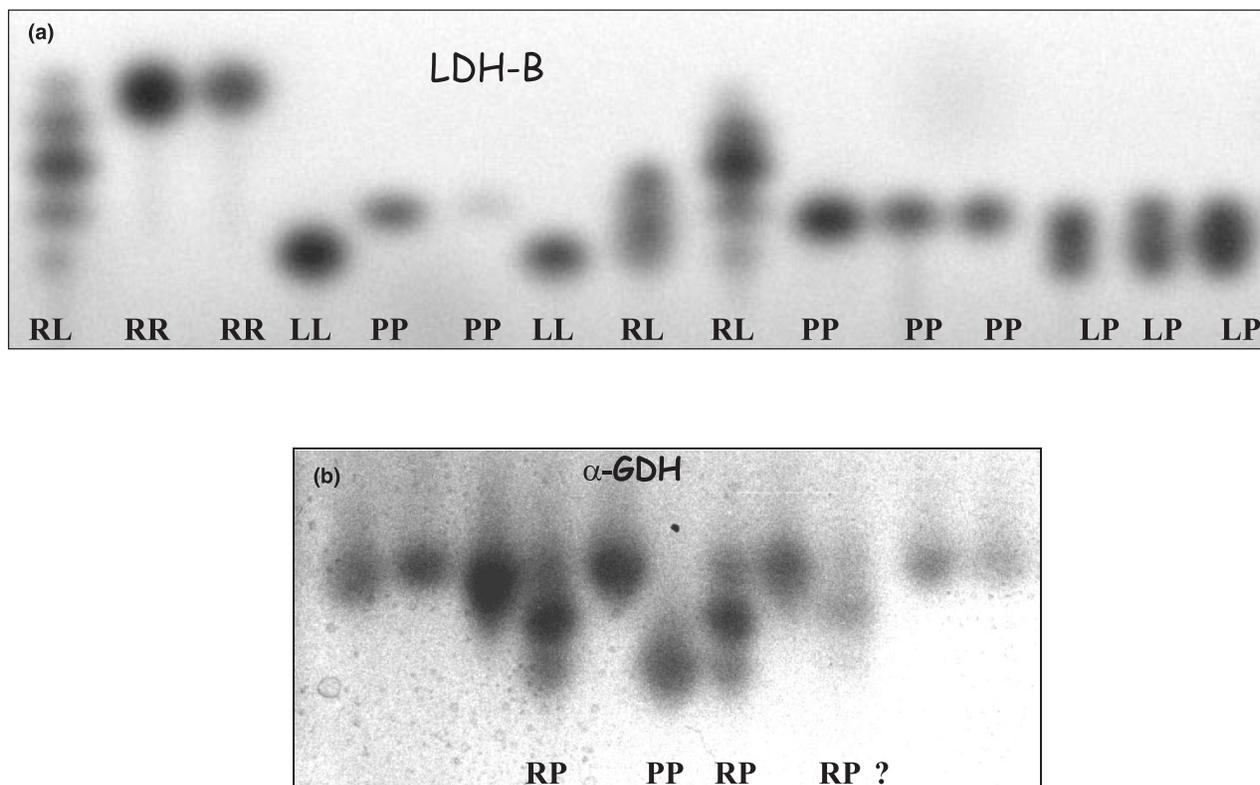


Fig. 2. Zymogrammes of different water frog taxa. RR, *R. ridibunda*; RL, *R. kl. esculenta*; LL, *R. lessonae*; PP, = *R. perezii*; LP, LP hybrids; RP, *R. kl. grafi*. (a) at the LDH-B locus, the faster allele (at the top of the figure) is specific of *R. ridibunda*, the slower of *R. lessonae*, the intermediate of *R. perezii*. Heterozygotes are, on this zymogramme, hybrids. (b) at the α-GDH locus, the slow allele (bottom of the figure) is specific of a *perezii* genome. With regard to other loci genotypes (especially the LDH one) it allows confirmation of the identification diagnostic

Among the 247 *R. ridibunda* frogs sampled, 114 were present in rivers or dead arms, 102 in gravel-pits, 28 in marshes, two in small ponds and one in a large pond. *Rana ridibunda* was more likely to occur in gravel pits, river and dead arms than in other habitats ( $\chi^2 = 242$ ;  $p < 0.0001$ ).

Among the 36 sites, the taxonomic composition of the frog populations corresponds to pure populations or stable hybridogenetic systems in 23 ponds (63.9% of the ponds) whereas it represents 'nonpredictable' populations, such as assemblages, in nine ponds (25%) or pure hybrid populations in four ponds (11.1%).

**Discussion**

**A new contact zone**

Our study revealed the existence of a previously undescribed contact zone between *R. lessonae* and *R. perezii* in South-

western France, where new hybridization events occurred (Fig. 1). These taxa mated and reproduced in the field in at least one pond and gave rise to offspring (that we 'named' LP hybrid tadpoles, i.e. individuals containing both a *perezii* and a *lessonae* genome). The 19 LP tadpoles caught in the field at the same pond were reared in the laboratory. They died before metamorphosis suggesting that such hybrids are not fully viable. However, several crossing experiments have given rise to viable offspring (Berger et al. 1995). Further investigations are necessary to assess to what extent hybridization between *R. lessonae* and *R. perezii* occurs in the wild, and whether LP hybrid tadpoles are indeed viable (and fertile as the hybridogens are). This sympatric area is located far from all previously known localities of *R. lessonae* (Graf and Polls-Pelaz 1989, Pagano et al. 2001). It can result from introduction or represent a relic distribution of this central European species. More data are needed to determine the extent of the distribution range of

Table 3. Allozymes and genotypes recorded among water frogs. The LDH-B allowed taxonomic identification of the whole taxa (see Fig. 2a). As a validation, MPI and PGM-2 locus allowed to discriminate between *lessonae* and *ridibunda* genome whereas CK-A, AHH and  $\alpha$ -GDH differentiated *perezi* genome from others (Fig. 2b)

LDH-B	MPI	PGM-2	$\alpha$ -GDH	CK-A	AHH	Species
a, c	a, c	b, d	Fast	a, b	Fast	<i>R. ridibunda</i>
ae, rarely ab, bc, ce	ah, ch	cd	Fast	a, b, ab	Fast	<i>R. kl. esculenta</i>
ad, rarely cd	al	dd	Fast-slow	ad	Fast-slow	<i>R. kl. grafi</i>
e, rarely b	h	c	Fast	a	Fast	<i>R. lessonae</i>
de	hl	cd	Fast-slow	ad	Fast-slow	LP hybrids
d	l	d	Slow	d	Slow	<i>R. perezi</i>

One letter, allozyme; two letters, genotype.

*R. lessonae* in the South-Western part of France and so of its sympatric occurrence with *R. kl. grafi* and *R. perezi*.

### New assemblages

Our study also revealed the existence of assemblages (*sensu* Pagano et al. 1997) never described such as the occurrence of *R. ridibunda* with *R. kl. grafi* (assemblage R-G), or with *R. perezi* (assemblage R-P), or with both *R. kl. grafi* + *R. perezi* (assemblage P-R-G). It is not possible to specify if such R-G assemblages are persisting hybridogenetic systems (analogous to R-E systems; Uzzell and Berger 1975) or whether they merely represent an assemblage (analogous to the R-E assemblage; Pagano et al. 1997). In fact, documenting the existence of a system or an assemblage requires to find out if genome exclusion occurs in the germinal cells of hybrids belonging to the specific population (Pagano et al. 1997).

The disparate distribution of these new assemblages (Fig. 1), mainly involving *R. ridibunda* individuals, suggests that their origin is often a direct consequence of the importation of frogs. Indeed, the distribution of *R. ridibunda* has been considered to be limited to the north-eastern areas of France (Graf and Polls-Pelaz 1989). Moreover, among the *R. ridibunda* populations present in Southern France, allochthonous origin has been documented in several populations (Pagano et al. 1997). In one case (Toulouse), the *R. ridibunda* involved in this R-G assemblage exhibited an 'allochthonous' allele (MPI-j; Pagano et al. 1997). As frog importation is still currently important in France, we can predict that similar assemblages will be found in other sites.

Although *R. ridibunda* exhibits preferences for river, dead arms and gravel pits, it may be found in other kinds of habitats (marshes, shallow ponds, large ponds) evidencing plasticity in habitat use. Thus, it offers opportunity for co-occurrence with other taxa such as *R. kl. grafi* (as in Salins de Badons and Orx) that occupy preferentially marshes (Pagano et al., 2001).

### Are population dynamics different in these new assemblages?

Considering male courtship call features, water frog evidence imperfect specific mate recognition system thus suggesting that hybridization events are numerous (Lodé and Pagano, 2000). In this context, how do frogs from different taxa mate in these new assemblages? *Rana kl. grafi* females may have the choice in mating either with their 'owns' males, or with *R. ridibunda* ones (in the R-G assemblage, at St Mathieu de Tréviers), or with *R. perezi* males plus the latter males (in the P-R-G assemblage at Salins de Badons). A question that remains is whether the P-R-G and R-P assemblages offer opportunities for 'primary' hybridization events (between *R. perezi* and *R. ridibunda*)? Our data do not allow us to answer such

questions, but it should be noted that assemblages represent 25% of the sites we sampled.

The hybridogens *R. kl. grafi* and *R. kl. esculenta* are considered as sexual parasites of their parental species *R. perezi* and *R. lessonae*, respectively (Graf and Polls-Pelaz 1989). The high rate of assemblage observed suggests that sexual parasitism of hybridogens may be more extended than previously thought (i.e. the hybridogen *R. kl. grafi* may be able to parasitize several sexual hosts such as *R. perezi* and *R. ridibunda*).

### *R. kl. grafi* origin

As the origin of *R. kl. grafi* is still unclear, the existence of a R-P assemblage suggests that this hybridogen may have originated from a primary hybridization occurring within this assemblage (assuming that females are present in such assemblages). However, we cannot discard the possibility that *grafi* hybridogens may arise from a primary hybridization between *R. perezi* and *R. kl. esculenta* (although we didn't record any P-E assemblage).

Pure persisting *R. kl. esculenta* populations or population where hybridogens are the main taxa have been described (Günther et al. 1979). In such cases, the progeny of hybrid-hybrid (*esculenta-esculenta*) mating exhibit a female excess (Berger et al. 1988). The female excess observed in Pissevache pond suggests such hybrid-hybrid matings between *R. kl. grafi*. Further investigations may specify (i) if pure *R. kl. grafi* populations exist; (ii) whether they may persist; and (iii) if such matings may generate *R. kl. grafi* triploids.

### Evolutionary perspectives

Due to the putative presence of *R. ridibunda* individuals of Balkan origin that are known to be resistant to hybridogenesis (Hotz and Uzzell 1983; Guerrini et al. 1997), the hypothesis of non-hybridogenetic events needs to be addressed. Thus, non-hybridogenetic hybrids may occur in natural populations. The fact that hybridogens are theoretically not affected by introgression processes suggests that the high rate of recombination observed in Southern France (Pagano and Schmeller 1999; Schmeller 1999) may be explained by the existence of hybrid, but non-hybridogenetic, populations in Southern France.

Schmidt (1993) proposed a model where water frogs may combine sexuality and asexuality. Asexuality characterizes hybridogenesis. The model of cyclical sexuality has been proposed because recombination and genetic exchanges are possible through hybridogen-hybridogen matings (Hotz et al. 1992; Schmidt 1993). In addition to this model, we proposed that introgressive hybridization is another possibility to maintain sexual reproduction, i.e. genetic exchanges. As Muller's ratchet is predicted to slow down when recombina-

tion occurs (Charlesworth et al. 1993), then, the prediction of a water frog evolutionary dead-end road (Milinski 1994) may be premature.

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## Zusammenfassung

*Neue Kontaktzonen und Gruppierungen bei Wasserfröschen in Südfrankreich*

Wasserfrösche sind vielfach an der Bildung von hybridogenetischen Komplexen beteiligt. Unsere Untersuchungen in Süd- und Westfrankreich zeigten jedoch neue Hybridisierungen zwischen *Rana lessonae* und *Rana perezi* (einige neue Hybride konnten im Kaulquappenstadium entdeckt werden) und das Bestehen von neuen Komplex-Gruppierungen [R-G, L-P, R-P, P-R-G: (R, *ridibunda*; G, *grafi*; L, *lessonae*; P, *perezi*)]. In Bezug auf den Hybridogenese-Mechanismus lassen diese Komplex-Gruppierungen sowohl primäre als auch sekundäre Hybridisierungen zu. Diese Besonderheiten können entweder durch Introgression oder durch das Bestehen von Reliktpopulationen erklärt werden; sie könnten so die Evolution der Wasserfrösche merklich beeinflussen.

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