Mixed breeding system in the hermaphroditic land slug Arion intermedius (Stylommatophora, Arionidae)

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Theory suggests that hermaphroditic plants and animals should be either entirely outcrossing or entirely selfing. As such, very few hermaphroditic plants and basommatophoran snails have a mixed breeding system. However, reliable estimates of selfing rates are lacking for most hermaphroditic animals. This partly prevents to delineate the relative contributions of the selective factors that determine selfing and outcrossing rates in hermaphroditic animal taxa. Here, we studied the population genetic structure of, and breeding system in, 11 populations of the hermaphroditic land slug *Arion intermedius* using five polymorphic microsatellite loci. Moreover, genotype frequencies deviated significantly from Hardy–Weinberg equilibrium expectations for most of the loci in all populations suggesting some level of selfing. Estimates of the selfing level *s*, suggest moderate levels of outcrossing (mean *s* based on $F_{15} = 0.84$; mean *s* based on the two-locus heterozygosity disequilibrium = 0.20, or with a ML approach = 0.22). Our study therefore suggests that *A. intermedius* has a mixed breeding system. A re-analysis of allozyme data from another arionid slug (subgenus *Carinarion*) indicates that mixed breeding may be more common in arionid slugs than hitherto was assumed. These results seem therefore at variance with current theoretical and empirical predictions and opens perspectives for the study on the evolutionary factors driving mixed breeding systems in animals.

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Mixed breeding, in which hermaphroditic species reproduce by both self- and cross-fertilization, is a challenging problem for evolutionary biologists (reviewed by GOODWILLIE et al. 2005; BARRETT 2013 and WRIGHT et al. 2013 for plants and ESCOBAR et al. 2011 for animals). Theory suggests that inbreeding depression should result in strategies of pure outcrossing or pure selfing, but under some assumptions mixed breeding systems are evolutionary stable (GOODWILLIE et al. 2005; WINN et al. 2011; but see IGIC and BUSCH 2013). In plants, predominant outcrossing and predominant selfing are common, while only few species have a mixed breeding system (VOGLER and KALISZ 2001; GOODWILLIE et al. 2005). In contrast, many animal species appear to have a mixed breeding system (JARNE and AULD 2006). However, estimates of the selfing rate in hermaphroditic animals are scarce and are mostly based on the fixation index $F_{\rm IS}$. Unfortunately, the use of F_{IS} may result in upward biases of the selfing rate, especially in outcrossing species, because most studies used microsatellites that may show null-alleles and scoring problems (e.g. stuttering, large allelic drop-out) (DAVID et al. 2007; JARNE and DAVID 2008). Indeed, using a more robust method to estimate selfing rates based on microsatellites, DAVID et al. (2007)

showed that hermaphroditic basommatophoran snails lack individuals that have a mixed breeding system (Escobar et al. 2011). This is in line with behavioural studies showing that, although many outcrossing hermaphroditic gastropods may be able to self (Jarne and DeLAY 1991; JARNE et al. 1993), 1) natural populations appear primarily or entirely outcrossing (JARNE and STÄDLER 1995), 2) offspring produced by isolated individuals suffer from strong inbreeding depression (Escobar et al. 2011), and 3) individuals immediately switch from self-fertilization to outcrossing when provided with a partner (reviewed by JORDAENS et al. 2007). Other species appear to be strictly self-fertilizing and suffer from strong outbreeding depression (Escobar et al. 2011).

Estimates of selfing levels in hermaphroditic gastropods are strongly biased towards the Basommatophora (17 species), while selfing rates in Stylommatophora (land snails and slugs) are only known for a few species (reviewed by ESCOBAR et al. 2011). However, there is circumstantial evidence of substantial variation in breeding systems in Stylommatophora as well. A case in point is the slug genus *Arion* (family Arionidae). Breeding experiments and allozyme studies have suggested that arionids may reproduce by exclusive outcrossing to exclusive self-fertilization (WILLIAMSON 1959; McCracken and Selander 1980; Foltz et al. 1982, 1984; SELANDER and OCHMAN 1983; BACKELJAU et al. 1997; JORDAENS et al. 2000). Until recently, one species, viz. Arion intermedius NORMAND, 1852 was suspected to be such an exclusive selfer because 1) none of the population genetic studies detected heterozygote individuals, but instead populations were either fixed for single homozygous multilocus genotypes (strains) or consisted of two or more of such strains (McCRACKEN and SELANDER 1980; FOLTZ et al. 1982; BACKELJAU and DE BRUYN 1991; BACKELJAU et al. 1992); 2) A. intermedius has never been observed to mate (QUICK 1960; CHICHESTER and GETZ 1973; DAVIES 1977); and 3) isolated specimens easily reproduce uniparentally for several generations without apparent strong inbreeding depression (Chichester and Getz 1973; Davies 1977). The first doubt as to whether A. intermedius is a strict selfer arose when GARRIDO et al. (1995) found spermatophores in the bursa copulatrix of Spanish specimens. Since spermatophores are produced during courtship (TOMPA 1984) and get into the bursa only by transfer from a mating partner (WIKTOR 1987), the occurrence of spermatophores suggests that specimens have mated. However, exchanging allosperm does not necessarily imply that this allosperm will also effectively fertilize the recipient's eggs. So, the mere observation of spermatophores is insufficient evidence to decide about the breeding system (BACKELJAU and DE BRUYN 1990). REISE et al. (2001) studied the population structure of 18 populations of A. intermedius using allozymes. Apart from two specimens from Jauernick-Buschbach (Germany), each containing a complete spermatophore, they also found eight heterozygotes in this population. Assuming that A. intermedius does not reproduce parthenogenetically (SELANDER and OCHMAN 1983; TOMPA 1984; RUNHAM 1993), the occurrence of heterozygote individuals in this population provided the first strong indication of some degree of outcrossing in A. intermedius. However, the sample sizes, as well as the numbers of allozyme loci surveyed and their degree of variability were too low to reliably estimate selfing rates (REISE et al. 2001). Therefore, we here re-assess the breeding system and population genetic structure of A. intermedius by screening five polymorphic microsatellite loci in 11 natural populations.

MATERIAL AND METHODS

Collection of slugs and microsatellite genotyping

A total of 227 adult *Arion intermedius* specimens were collected by hand in ten European and one North American

populations (Table 1). Animals were stored in absolute ethanol. Genomic DNA was extracted from individual foot muscle tissue according to PINCEEL et al. (2004). Samples were genotyped at six microsatellite loci (Arin1 to Arin6) following BROOKES et al. (2001), but because Arin6 yielded bad PCR results, this locus was excluded from further analyses. PCR products were electrophoresed using an automated ALFexpress DNA Sequencer (Amersham Pharmacia Biotech). The program ALLELELINKS ver. 1.00 (Amersham Pharmacia Biotech) was used to determine allele size. Individuals with known allele sizes and size standards were included on each gel to ensure scoring accuracy and consistency among gels.

Population genetic analyses

Overall population level genetic diversity was quantified as the mean number of alleles per locus (MN_A) , observed heterozygosity (H_0) (i.e. the mean proportion of heterozygotes at a locus) and NEI's (1978) unbiased expected heterozygosity $(H_{\rm E})$ using the program GENETIX ver. 4.05.2 (BELKHIR et al. 2000). Allelic richness (AR) for each locus and for each population was estimated with FSTAT ver. 2.9.3.2 (GOUDET 2005; available at <www2. unil.ch/popgen/softwares/fstat.htm>). Allelic richness is a measure of the number of alleles independent of sample size, hence allowing to compare this quantity between different sample sizes (EL MOUSADIK and PETIT 1996). The estimator of single and multilocus F_{IS} values, f (WEIR and COCKERHAM 1984) (also called the fixation index or inbreeding coefficient), was estimated with FSTAT ver. 2.9.3.2 (GOUDET 2005). F_{IS} measures the reduction of heterozygosity within populations based on a correlation of alleles within individuals (CROW and KIMURA 1970, WEIR and COCKERHAM 1984). When defined as $1 - H_0 / H_E$ it can be used to measure the extent to which a particular population departs from Hardy-Weinberg equilibrium (HWE) expectations. Deviation of genotype frequencies from HWE expectations for each locus and population was analysed with an exact test using GENEPOP ver. 4.0 (ROUSSET 2008). Self-fertilization is the most extreme form of inbreeding and thus high values of F_{IS} (or significant heterozygote deficits) may be indicative of self-fertilization (JARNE 1995).

Populations were also screened for private alleles (N_{PA}), i.e. alleles that occur in only one population. Linkage disequilibrium (LD) between pairs of loci was tested for each population using GENEPOP ver. 4.0 (ROUSSET 2008). LD measures the significance of association between genotypes at pairs of loci in each population. If selfing levels are high, the decay of LD is slowed down substantially because inbreeding reduces the number of double heterozygotes by which LD is eliminated (NORDBORG 2000). GENEPOP was further used to assess heterogeneity

Table 1. List of populations and numbers of specimens sampled (N) in eleven populations of the hermaphroditic land slug Arion intermedius. Genetic diversity was quantified as: mean number of alleles per locus (MN_A) , allelic richness (AR), number of private alleles (N_{PA}) , NEI's (1978) gene diversity (H_E) , observed heterozygosity (H_O) . The selfing rate s was estimated by three methods: F_{1S} , \hat{g}_2 and ML (DAVID et al. 2007). Detailed information on the different measures is given in the text.

	Geneti	c diversity						Selfin	ng rate
Country	N	MN_A	AR	N_{PA}	$H_{\scriptscriptstyle E}$	H_{O}	$F_{\rm IS}$	\hat{g}_2	ML
Belgium									
Huy	21	2.8 ± 0.9	2.21	1	0.26 ± 0.10	0.06 ± 0.04	0.81	0	0.18
Ekeren	15	2.0 ± 0.4	1.87	0	0.28 ± 0.14	0.02 ± 0.02	0.97	_	_
France									
Rambouillet	20	3.2 ± 0.6	2.55	2	0.36 ± 0.12	0.06 ± 0.02	0.86	0	0.08
Le Landin	16	2.6 ± 0.4	2.34	0	0.35 ± 0.06	0.13 ± 0.05	0.69	0.90	0.84
Neufchatel	21	3.0 ± 0.4	2.55	0	0.40 ± 0.11	0.14 ± 0.06	0.75	0.23	0.37
United Kingdom									
Bilsington	19	2.4 ± 0.4	2.18	0	0.39 ± 0.10	0.11 ± 0.03	0.84	0	0.07
Rugeley	24	2.6 ± 0.4	2.01	1	0.26 ± 0.10	0.01 ± 0.01	0.99	_	_
Lamswick	25	4.0 ± 1.0	2.86	4	0.47 ± 0.13	0.05 ± 0.03	0.94	0	0.11
Beeding	30	3.4 ± 0.8	2.69	3	0.41 ± 0.14	0.18 ± 0.14	0.76	0.44	0.10
Portugal (Madeira)									
Serra de Água	20	3.2 ± 0.4	2.70	1	0.55 ± 0.03	0.26 ± 0.10	0.72	0	0.03
USA									
Barnstable	16	2.6 ± 0.5	2.04	1	0.27 ± 0.09	0.02 ± 0.02	0.94	_	_

in allele frequencies ("genic differentiation"; whether the allelic distribution is identical across populations) and genotype frequencies ("genotypic differentiation"; whether the genotypic distribution is identical across populations) among populations using an unbiased estimate of Fisher's exact test (ROUSSET 2008).

Selfing rates (s) were estimated by three approaches: 1) from the inbreeding coefficient (f) using the classical formula $\hat{s}(f) = 2f/(1+f)$, 2) from two-locus heterozygosity disequilibrium (\hat{g}_2) , using the program RMES (DAVID et al. 2007; available at < www.cefe.cnrs.fr/en/genetiqueet-ecologie-evolutive/patrice-david>), and 3) using a maximum likelihood (ML) approach by maximizing the log-likelihood (LnL) of the multilocus heterozygosity structure of the sample, also with RMES. Yet, RMES could not be applied to the populations from Rugeley, Ekeren and Barnstable because these populations yielded heterozygotes only at single loci. Sequential Bonferroni corrections were applied throughout (RICE 1989). Null alleles may bias F_{IS} estimates upward (DAKIN and AVISE 2004; DAVID et al. 2007; JARNE and AULD 2006). Therefore, we estimated theoretical mean null allele frequencies (and their standard error) with the program FREENA (CHAPUIS and ESTOUP 2007).

RESULTS

A total of 38 alleles were detected at the five microsatellite loci. Thirteen alleles were unique to single populations ($N_{PA} = 13$). More than one private allele was found in Lamswick (four), Beeding (three) and Rambouillet (two). MN_A values ranged between two and four, while H_0 and H_E values varied, respectively, between 0.01 and 0.26 and between 0.26 and 0.55 (Table 1). There was no significant correlation between sample size and H_0 (Spearman R = 0.10; N = 10; P = 0.78), H_E (Spearman R = 0.35; N = 10; P = 0.30) or allelic richness (Spearman R = 0.52; N = 10; P = 0.10) suggesting little, if any, sampling bias on genetic diversity estimates.

There was only significant LD in five out of 80 tests, but these were not significant after Bonferroni correction. The populations from Serra de Água and Lamswick were genetically the most diverse, both in MN_A , AR and H_E (Table 1). There was significant genic and genotypic differentiation among populations at all loci (all P < 0.0001), even after Bonferroni correction. Especially the populations of Serra de Água, Beeding and Barnstable were well differentiated from the other populations. The Serra de Água population had a private allele at Arin5 (allele C) with a very high frequency (0.528; Table 2). Similarly, a private allele at Arin3 was found in the Beeding population (allele B). Both populations also had a high frequency for the A allele at Arin3 (respectively 0.25 and 0.28) that was absent in the other populations. Significant departures from HWE (i.e. heterozygote deficits) were observed at all loci in many populations, even after Bonferroni correction (Table 2). Nevertheless, heterozygotes were observed in all populations and several H_0 values were relatively

	Belg	ium		France			D	K		Portugal	USA
Locus	Huy	Ekeren	Rambouillet	Le Landin	Neufchatel	Bilsington	Rugeley	Lamswick	Beeding	Serra de Água	Barnstable
ARIN1											
(N)	10	11	15	13	20	8	15	13	12	16	11
A	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.083	0.000	0.000
В	0.000	0.000	0.067	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
C	0.000	0.000	0.000	0.000	0.425	0.000	0.000	0.000	0.042	0.063	0.000
D	0.000	0.000	0.167	0.231	0.425	0.000	0.000	0.000	0.000	0.063	0.000
Ц	0.100	0.000	0.033	0.077	0.000	0.313	0.000	0.000	0.000	0.250	0.000
Ц	0.700	0.091	0.733	0.038	0.075	0.688	0.933	0.538	0.875	0.625	0.273
IJ	0.200	0.909	0.000	0.654	0.075	0.000	0.067	0.462	0.000	0.000	0.727
$F_{\rm IS}$	1.000	1.000	0.854	0.860	0.848	0.741	1.000	1.000	0.656	0.333	1.000
HWE	*	0.048	*	*	*	0.077	0.035	*	0.044	0.010	0.002
ARIN2											
(Z)	11	11	15	14	18	17	22	24	26	20	18
Α	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.045	0.000	0.000	0.000
В	0.000	0.045	0.000	0.000	0.000	0.000	0.000	0.021	0.750	0.000	0.028
C	0.091	0.000	0.000	0.000	0.111	0.029	0.000	0.021	0.000	0.025	0.000
D	0.909	0.591	0.233	0.214	0.194	0.500	0.955	0.458	0.154	0.350	0.083
Щ	0.000	0.364	0.767	0.786	0.694	0.471	0.000	0.396	0.096	0.625	0.861
ц	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.063	0.000	0.000	0.000
IJ	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.028
Н	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.042	0.000	0.000	0.000
$F_{\rm IS}$	-0.053	0.839	0.825	0.187	0.313	0.682	1.000	0.744	0.908	0.605	0.575
HWE	1.000	0.001	0.005	0.490	0.020	0.002	0.023	*	*	0.003	0.008
ARIN3											
(Z	16	9	12	7	18	13	23	19	25	16	19
А	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.280	0.250	0.000
В	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.400	0.000	0.000
C	0.063	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
D	0.125	0.000	0.000	0.000	0.000	0.000	0.000	0.053	0.000	0.000	0.000
Щ	0.063	0.000	0.083	0.000	0.000	0.000	0.000	0.105	0.000	0.000	0.000
ц	0.031	0.000	0.000	0.143	0.056	0.000	0.000	0.000	0.000	0.031	0.000
IJ	0.000	0.000	0.000	0.071	0.083	0.115	0.087	0.026	0.040	0.000	0.000
Н	0.688	0.500	0.333	0.786	0.611	0.115	0.348	0.289	0.060	0.656	0.211
I	0.031	0.167	0.167	0.000	0.250	0.769	0.522	0.368	0.180	0.063	0.684
											(Continued)

Table 2. (C	ontinued)										
	Belg	jium		France			Ū	X		Portugal	USA
Locus	Huy	Ekeren	Rambouillet	Le Landin	Neufchatel	Bilsington	Rugeley	Lamswick	Beeding	Serra de Água	Barnstable
ARIN3											
J	0.000	0.000	0.333	0.000	0.000	0.000	0.043	0.000	0.000	0.000	0.105
К	0.000	0.333	0.000	0.000	0.000	0.000	0.000	0.158	0.000	0.000	0.000
L	0.000	0.000	0.083	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Μ	0.000	0.000	0000	0.000	0.000	0.000	0.000	0.000	0.040	0.000	0.000
$F_{ m IS}$	0.765	1.000	1.000	0.647	0.905	0.813	1.000	0.933	0.947	0.883	1.000
HWE	*	0.004	*	0.077	*	0.001	*	*	*	*	*
ARIN4											
(Z)	18	6	17	11	16	16	22	19	25	20	20
Α	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.053	0.000	0.000	0.000
В	0.000	0.000	0.029	0.000	0.000	0.000	0.000	0.026	0.020	0.275	0.000
C	0.000	0.000	0.000	0.000	0.094	0.000	0.000	0.000	0.380	0.350	0.050
D	0.944	1.000	0.059	0.818	0.906	1.000	0.955	0.763	0.220	0.375	0.050
Е	0.056	0.000	0.912	0.182	0.000	0.000	0.045	0.105	0.380	0.000	0.900
н	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.053	0.000	0.000	0.000
$F_{ m IS}$	1.000		0.312	1.000	-0.071		1.000	0.875	-0.067	0.118	1.000
HWE	0.029		0.091	0.008	1.000		0.023	*	*	*	*
ARIN5											
(Z	6	9	16	13	16	11	23	18	26	18	20
A	0.000	0.000	0.031	0.077	0.000	0.091	0.152	0.000	0.000	0.000	0.000
В	1.000	1.000	0.969	0.923	0.938	0.636	0.761	1.000	1.000	0.472	1.000
C	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.528	0.000
D	0.000	0.000	0.000	0.000	0.063	0.273	0.087	0.000	0.000	0.000	0.000
$F_{ m IS}$				-0.043	1.000	0.672	0.893			0.894	
HWE				1.000	0.032	0.003	*			*	

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high (albeit significantly lower than the $H_{\rm E}$ values) in the populations of Serra de Água, Beeding, Le Landin and Neufchatel (Table 2).

The average selfing rate over all populations was relatively high using the estimate based on F_{IS} values (s = 0.84; range 0.69 – 0.99), but generally lower with the more robust estimates \hat{g}_2 (s = 0.20; range 0.00 – 0.90) and ML (s = 0.22; range 0.03 – 0.84). (Table 1). Null allele frequencies differed strongly among loci and were low for Arin4 and Arin5 ($9.0 \pm 3.8\%$ and $10.0 \pm 3.4\%$, respectively), but higher for Arin2 ($17.0 \pm 3.1\%$), Arin1 ($24.7 \pm 2.3\%$) and Arin3 ($33.1 \pm 2.3\%$).

DISCUSSION

The present study shows that populations of the terrestrial slug Arion intermedius show substantial population genetic differentiation, both in the frequency of alleles and genotypes, as in the amount of genetic diversity. Significant population differentiation is also found in other arionid species (ENGELKE et al. 2011) and can be explained by a variety of (historical and recent) factors and processes. Even though it is difficult to disentangle the relative contributions of all of these (discussed by ENGELKE et al. 2011) the breeding system is probably the most important factor (JARNE 1995). Unfortunately, reliable estimates of the relative occurrence of selfing and outcrossing in hermaphroditic animals are currently only available for basommatophoran gastropods where species either appear almost entirely self-fertilizing or strongly outcrossing (Escobar et al. 2011).

Populations of A. intermedius displayed significant departures from HWE with strong heterozygote deficiencies that were not locus-specific In hermaphrodites, this is considered to be indicative for the occurrence of selfing. Yet, some loci showed substantial null allele frequencies and these cause F_{IS} estimates, and consequently selfing estimates, to be biased upward (DAKIN and AVISE 2004; DAVID et al. 2007; JARNE and AULD 2006). Yet, when correcting estimates of the selfing level for the presence of null alleles, selfing levels in A. intermedius still vary between 0 (complete outcrossing) and 0.90 (predominant selfing). Hence, there seems considerable variation in the amount of selfing among populations of A. intermedius and this strongly suggests that this species is not a strict selfer (see also REISE et al. 2001). Consequently A. intermedius is a species with a mixed breeding system that may be an exception to the general rule that hermaphroditic species should either be strict selfers or strict outcrossers (GOODWILLIE et al. 2005; ESCOBAR et al. 2011).

Similarly, using variation at allozyme data JORDAENS et al. (2000) showed variation in the selfing rate among populations in land slugs of the arionid subgenus *Carinarion*. F_{B} -based selfing rates varied geographically with high selfing rates (up to 1) in northeastern European populations and low selfing rates (up to 0.315) in some central European populations. A re-analysis of these allozyme data using the ML method of DAVID et al. (2007) resulted in selfing rates of s = 0 to 1 and confirm our previous findings that *Carinarion* has a mixed breeding system too. Also PRÉVOT et al. (2013) found substantial interpopulation variation in the selfing level (*s* estimated from nine polymorphic microsatellite loci by ML varied from 0 to 0.817) in the land snail *Rumina decollata* in southern France.

Several factors, including the magnitude of in- and outbreeding, population density, the probability of finding mates, the life history and seasonal changes (see JARNE and Charlesworth 1993, Jarne and Auld 2006 and Esco-BAR et al. 2011 for a more extensive list) may influence selfing rates. Given the among-population variation in selfing rates in A. intermedius (this study) and Carinarion (JORDAENS et al. 2000), these, and other species of the genus Arion (WILLIAMSON 1959, HAGNELL et al. 2006, ENGELKE et al. 2011), and R. decollata (SELANDER and KAUFMAN 1973, Prévot et al. 2013) may be very suitable stylommatophoran taxa to study the various aspects listed above, in the same way as Planorbidae or Physidae are for the Basommatophora. Moreover, the list of other stylommatophoran species that are probably capable of both outcrossing and self-fertilization (but in which reliable estimates of the outcrossing or selfing level are lacking) is substantial, viz. Cochlicopa spp. (Armbruster and SCHLEGEL 1994), Deroceras laeve (HOFFMANN 1983), Deroceras praecox (REISE 1996), Deroceras juranum (REISE 1997), Zonitoides nitidus (JORDAENS et al. 1998), Vertigo pusilla (POKRYSZKO 1990), Chondrina clienta (BAUR and KLEMM 1989) and Balea perversa (WIRTH et al. 1997). Hence, stylommatophoran land snails may offer ample opportunities to study the evolution of mixed breeding in hermaphroditic animal species.

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