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Variation in inter-simple sequence repeat (ISSR) in mangrove and non-mangrove populations of *Heritiera littoralis* (Sterculiaceae) from China and Australia

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Abstract

Genetic diversity within and among 10 mangrove and non-mangrove populations of *Heritiera littoralis* from three sites in China and one site in Australia was determined using inter-simple sequence repeats (ISSR). Eleven primers produced 173 bands across a total of 166 individuals. At the species level, genetic diversity was high ($P = 93.1\%$, He $= 0.24$, and $I = 0.37$). Higher genetic diversity was observed in Australian populations compared to Chinese populations, which can be explained by either ecogeographic variation or evolutionary history. A significant correlation between genetic and geographic distances was detected among the 10 populations, but no such correlation was found among either Chinese or Australian populations. AMOVA indicated that 41.7% of the total genetic variation was explained by differences between China and Australia. As for Chinese populations, 22.5% of the genetic variation was accounted for by geographical effects with 5.3% for differentiation between habitats. We suggest that geographic distance is the primary cause of genetic differentiation between China and Australia at the regional scale, whereas, the strongly divergent habitats (marine versus terrestrial) also play an important role in within region differentiation at the local scale. An understanding of the genetic structure of *H. littoralis* provides insight for the conservation and the management of these species.

Keywords: *Heritiera littoralis*; Genetic diversity; Genetic structure; ISSR; Mangrove

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1. Introduction

Mangrove forests characterize tropical and subtropical coastlines worldwide. It is evident that the floristically richest mangrove regions are Indo-Malayan; moreover, Australia is a secondary center of mangrove diversity (Saenger, 1998). Despite their ecological and economic importance, mangroves are being destroyed worldwide at an alarming rate (Farnsworth and Ellison, 1998). In China, the area occupied by the mangrove forests has diminished to 15,000 ha from the original 50,000 ha since the 1950s (Zhang and Sui, 2001). To overcome these losses, China plans to plant 60,000 ha of mangrove forests along the southern coastal areas in the coming decade as part of a state project to build up the coastal shelterbelt (http://www.fpeng.peopledaily.com.cn/200202/02/eng20020202_89802.shtml).

Genetic diversity is critical for adaptation to environmental changes and for long-term survival of a species. Knowledge of genetic diversity within and among populations is practically important for conservation management. For instance, the genetic diversity within donor populations and the genetic similarity between donor and recipient populations are crucial factors to successful restoration (Hamrick and Godt, 1996). In order to have effective conservation strategies, population genetic studies of mangroves are essential for providing information needed to conserve genetic resources as well as to evaluate afforestation, domestication, and breeding programs. So far, such studies of mangroves mainly have dealt with either *Avicennia* (Maguire et al., 2000) or four genera in tribe Rhizophoreae (Rhizophoraceae), i.e., *Rhizophora* (Duke et al., 2002), *Ceriops* (Ge and Sun, 2001), *Bruguiera* (Abeysinghe et al., 2000), and *Kandelia* (Sun et al., 1998). The extent and patterns of genetic diversity in other mangrove species are still largely unknown.

The looking-glass mangrove, *Heritiera littoralis* Dryand (Sterculiaceae), occurs from eastern Africa to southern Asia, Australia, and Melanesia (Tomlinson, 1986); introduced further east to Hawaii and Tonga (Kostermand, 1959). This mangrove tree may be found in both riverine and marine environments. However, *H. littoralis* has declined rapidly due to over-exploitation for timber, fuel, and fodder (Das et al., 2001), and it is now difficult to find distinct zones dominated by this species. Individuals occur on riverbanks, along tidal estuaries, and at the most landward fringe of mangrove swamps, sometimes intermix with terrestrial vegetation. Detailed studies of this species, including physiological, cytological, cytochemical, and molecular aspects, are largely lacking. Recently, Das et al. (2001) applied RAPD markers and nuclear DNA content to infer phylogenetic relationships within *Heritiera*, and found that *H. littoralis* was closer to its mangrove congener *Heritiera fomes* than to the non-mangrove *Heritiera macrophylla*.

Inter-simple sequence repeat (ISSR) analysis involves the polymerase chain reaction (PCR) amplification of regions between adjacent, inversely oriented microsatellites using a single simple sequence repeat (SSR)-containing primer (Zietkiewicz et al., 1994). Compared with the widely used RAPD markers, ISSR technique uses longer primers allowing for higher annealing temperatures that result in greater reproducibility of the bands (Wolfe and Liston, 1998). Because of the high polymorphism, ISSRs have been employed successfully in population genetic studies in many cultivated and wild plants (Huang and Sun, 2000; Esselman et al., 1999). ISSRs also have proven useful in evaluating genetic diversity in the mangrove species *Aegiceras corniculatum* and *Ceriops tagal* (Ge and Sun, 1999, 2001).

In this study, we applied ISSR markers to investigate genetic variation within and among natural populations of *H. littoralis* growing in mangrove and non-mangrove habitat from China and Australia. The following questions are addressed: (1) What is the population genetic diversity of *H. littoralis* and how efficient are ISSR markers in studying this species? (2) Is there any genetic differentiation between populations originating from China and Australia, among populations from different regions, and among populations from different habitats within the same region? (3) Are there any implications of our findings for future conservation and breeding programs for this species?

2. Materials and methods

2.1. Sampling

A total of 166 individuals from 10 natural populations of *H. littoras* was sampled (Table 1). In China, three coastal areas, i.e., Xiangkeng, Yanzao, and Qinglan, along the south China sea were chosen for investigation which contained both mangrove and non-mangrove stands of *H. littoralis*, and can represent more than 70% of the range of this species in China. The three regions are separated from each other by varying distances: Xiangkeng to Yanzao ∼56 km, Yangzao to Qinglan ∼573 km, and Qinglan to Xiangken ∼520 km. Within each region, disjunct stands that grew either in mangrove swamps or in woodland areas were considered as operable populations in this study, since, their genetic structure was to be explored. In north-eastern Australia, the Daintree River, Queensland was chosen for study because of its riverine mangrove environment. Along the Daintree River, mangroves are dominant near the coast and are replaced gradually by a rainforest community farther upstream. Four distinct populations of *H. littoralis* were sampled along the Daintree River, ranging from inland to freshwater and to intertidal habitats, with an increasing degree of soil salinity. Leaf materials from 10 to 20 randomly selected trees were collected in each population at the intervals of at least 5 m. Chinese populations were sampled during June and October in 2001, and Australian populations were sampled in November 2001. Leaves were stored with silica gel in zip-lock plastic bags until DNA isolation.

2.2. DNA isolation and PCR amplification

Genomic DNA was isolated using the CTAB method (Doyle and Doyle, 1987). One hundred ISSR primers from the Biotechnology Laboratory, University of British Columbia (UBC set no. 9), were initially screened and 11 of the primers that produced clear and reproducible fragments were used: 808 $(AG)_8G$, 811 $(GA)_8C$, 814 $(CT)_8A$, 828 $(TG)_8A$, 835 $(AG)_8YC$, 841 $(GA)_8YC$, 848 $(CA)_8RG$, 856 $(AC)_8G$, 859 $(TG)_8RC$, 864 $(AT)_8G$, and 880 G $(GA)_8$. After quantification with a fluorometer (Hoefer), a working sample solution of 20 ng μl^{-1} DNA was prepared. Each 10 μl amplification reaction consisted of 1.5 mM $MgCl_2$, 250 μM dNTPs, 2% formamide, 0.25 μM primer, 1U Taq DNA polymerase, and 10 ng template DNA. Amplification was performed in a PTC-200 thermocycler under the following cycle profile: 5 min at 94 °C, followed by 45 s at 94 °C, 45 s annealing at 52 °C, and 1.5 min extension at 72 °C for 45 cycles, and 7 min at 72 °C for a final extension. The

Table 1
Location, habitat, sample size, and genetic variability of *H. littoralis* populations studied

Country	Location	Site co-ordinate	Population code	Habitat	Sample size	*P* (%)	He	*I*
China	Xiangkeng	22°48′N, 115°01′E	XK-1	Mangrove	19	42.2	0.122 ± 0.170	0.190 ± 0.250
			XK-2	Woodland	18	37.0	0.116 ± 0.176	0.177 ± 0.257
	Yanzao	22°38′N, 114°30′E	YZ-1	Mangrove	16	34.7	0.112 ± 0.174	0.171 ± 0.255
			YZ-2	Woodland	20	38.2	0.110 ± 0.169	0.170 ± 0.247
	Qinglan	19°37′N, 110°40′E	QL-1	Mangrove	19	43.4	0.139 ± 0.186	0.211 ± 0.269
			QL-2	Woodland	16	42.8	0.134 ± 0.182	0.205 ± 0.265
Australia	Daintree River	16°16′S, 145°18′E	DR-1	Brackish intertidal wetland	10	48.6	0.122 ± 0.162	0.196 ± 0.238
			DR-2	Fresh-brackish water border	13	61.3	0.179 ± 0.179	0.278 ± 0.259
			DR-3	Fresh water wetland	16	62.4	0.167 ± 0.169	0.265 ± 0.246
			DR-4	Inland	19	52.0	0.155 ± 0.187	0.238 ± 0.269
			Mean		16.6	46.3 ± 9.7	0.136 ± 0.024	0.210 ± 0.038
			At the species level		166	93.1 ± 25.5	0.236 ± 0.174	0.365 ± 0.233

P: the percentage of polymorphic loci; He: mean expected heterozygosity; *I*: Shannon's information index.

amplification products were electrophoresed on 2% agarose gels buffered with 0.5× TAE and detected by ethidium bromide staining. Gene Ruler 100 bp DNA ladder plus (Shengong Inc., Shanghai, China) was used to determine the size of the ISSR fragments.

2.3. Data analysis

ISSR bands were scored as present (1) or absent (0) for each DNA sample excluding the smeared and weak ones. The binary data matrix was input into POPGENE (Yeh et al., 1997), assuming Hardy–Weinberg equilibrium. The following indices were used to quantify the amount of genetic diversity within each population examined: the percentage of polymorphic loci (*P*), the mean expected heterozygosity (He) (Nei, 1973), and Shannon's information index of diversity (*I*). Genetic diversity parameters (*P*, He, and *I*) were also calculated at the species level.

Genetic differentiation among populations was estimated by Nei's gene diversity statistics (Nei, 1973). Gene diversity statistics were calculated for two regions, e.g., China and Australia, and for the populations overall. The amount of gene flow among these populations was estimated as $N_m = (1/G_{ST} - 1)/4$ (Slatkin and Barton, 1989). Pairwise genetic distance between populations (Nei, 1972) was calculated to construct a UPGMA dendrogram.

Analysis of molecular variance (AMOVA) was conducted to calculate variance components and their significance levels for variation between plants from China and Australia, among three distinct regions of China, among populations within a region, and within populations using AMOVA version 1.55 (Excoffier, 1993). The input files for AMOVA were prepared by the aid of AMOVA-PREP version 1.01 (Miller, 1998).

3. Results

3.1. ISSR profile

In preliminary studies, we examined the repeatability of bands by both repeating the ISSR process in its entirety and running the same PCR product twice in separate lanes across 20 samples. It proves that patterns of ISSR are highly reproducible. The 11 primers produced a total of 173 reproducible bands across all 166 individuals of the 10 populations. Of the 173 loci surveyed, 161 were polymorphic. The size of the ISSR fragments varied from 0.3 to 2.2 kb. Populations showed clear differentiation between China and Australia. Twenty loci present in the Chinese populations were absent in those from Australia. Two loci were fixed in all the Chinese individuals. Similarly, 54 loci were present in the Australia populations, but absent in those from China. Three of those loci were fixed in all the Australian individuals. In addition, each of the 166 individuals presented a unique ISSR genotype, indicating extensive genetic variation in the populations studied.

3.2. Genetic diversity and differentiation

At the species level, the percentage of polymorphic loci (*P*), mean expected heterozygosity (He), and Shannon's index (*I*) were 93.1%, 0.236, and 0.365, respectively (Table 1).

Table 2
Comparison of genetic diversity estimates in *H. littoralis* between different groups of populations as determined using ISSR markers

Parameters	% of polymorphic loci (*P*)	Total gene diversity (Ht)	Within population (Hs)	Between populations (Dst)	Coefficient of gene differentiation (G_{ST})	Gene flow (N_m)
Total	93.1	0.236 ± 0.030	0.136 ± 0.011	0.101 ± 0.020	0.426	0.336
China	57.2	0.160 ± 0.030	0.122 ± 0.018	0.038 ± 0.012	0.238	0.800
Australia	72.3	0.176 ± 0.027	0.156 ± 0.021	0.020 ± 0.006	0.115	1.923

The coefficient of gene differentiation was high ($G_{ST} = 0.426$). At the population level, the mean values of *P*, He, and *I* were 46.3%, 0.136, and 0.210, respectively. In particular, all three parameters indicated that genetic diversity within populations was higher in Australian populations than Chinese populations. When populations were grouped according to geographic region, genetic diversity of the Australian group was significantly higher than the Chinese group as revealed by both the percentage of polymorphic loci and the total gene diversity (Table 2). Populations in China exhibited higher genetic differentiation ($G_{ST} = 0.238$) than those in Australia ($G_{ST} = 0.115$). Based on G_{ST} values, the estimated number of migrants per generation (N_m) between populations in China and Australia was 1.923 and 0.8, respectively, with a much lower value of 0.336 for all 10 populations.

3.3. Cluster analysis

A UPGMA dendrogram based on pairwise genetic distances identified two geographic clusters, i.e., Chinese and Australian populations (Fig. 1). Within the cluster of Chinese populations, six populations formed three groups according to locality. Populations in Yanzhao, however, showed a closer genetic relationship to those in Qinglan than to those in Xiangkeng, the closer population geographically. Within the cluster of Australian populations, the intertidal population differed substantially from the other three populations. Furthermore, the two populations growing in the freshwater zone were more similar to each other genetically

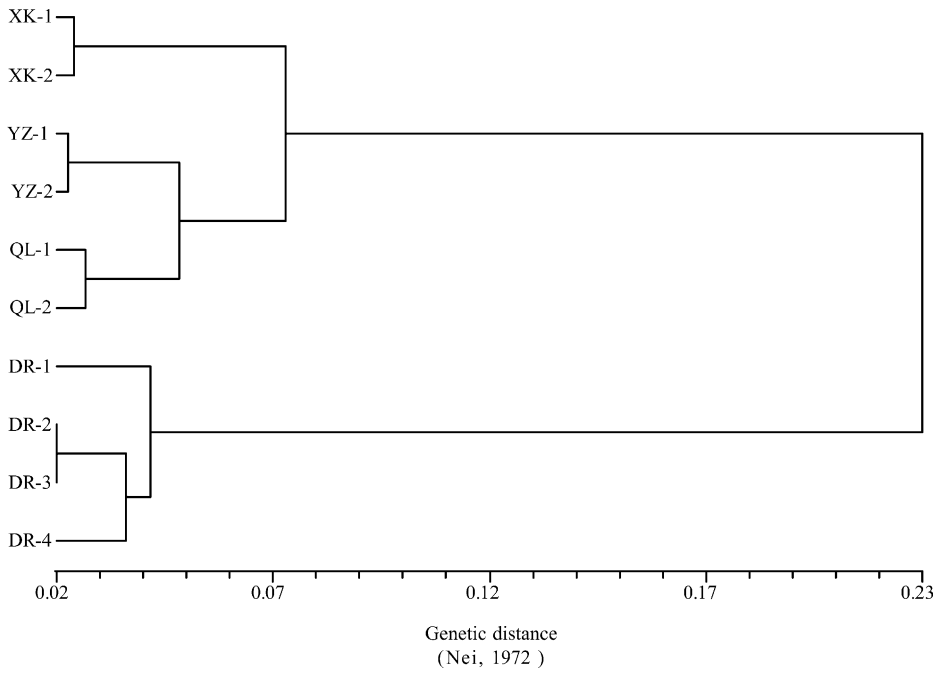

Fig. 1. UPGMA dendrogram of *H. littoralis* populations based on ISSR markers. Population codes are listed in Table 1.

Table 3
Summary of nested analysis of molecular variance (AMOVA) based on ISSR genotypes of *H. littoralis*

Source of variation	d.f.	S.S.D.	M.S.D.	Variance component		*P*
				Absolute	%	
A						
Among regions	1	1052.879	1052.879	12.993	41.7	<0.001
Among populations within regions	8	586.871	73.359	3.544	11.4	<0.001
Within populations	156	2281.093	14.622	14.622	46.9	<0.001
Total	165	3920.843	23.763	31.159		
B						
Among regions	2	346.885	173.443	3.992	22.5	<0.001
Among populations within regions	3	88.652	29.551	0.936	5.3	<0.001
Within populations	102	1307.065	12.814	12.814	72.2	<0.001
Total	107	1742.602	16.286	17.742		

Levels of significance are based on 1000 iteration steps. (A) Two regions: China vs. Australia, (B) three regions within China (Xiangkeng, Yanzao, and Qinglan). S.S.D.: sum of squared deviation; M.S.D.: mean of squared deviation.

than to the inland one. The Mantel test revealed that there was a statistically significant correlation between pairwise genetic distance and corresponding geographic distance among all 10 populations ($r = 0.978$, $P = 0.004$). However, further measures of the Mantel test comparing genetic and geographic distances among either the six Chinese populations ($r = 0.384$, $P = 0.062$) or the four Australian populations ($r = 0.728$, $P = 0.057$) were not statistically significant.

3.4. AMOVA analysis

Analysis of molecular variance indicated that almost half of the total variation (41.7%) was accounted for by differentiation between Chinese and Australian populations, with a further 11.4% accounting for variation among populations within regions, and the remainder (46.9%) partitioned among individuals within a population (Table 3A). When further partitioning variance among Chinese populations, we found that 22.5% could be explained by site and 5.3% by the two chosen habitats, e.g., mangrove and woodland (Table 3B). All components of molecular variance were highly significant ($P < 0.001$). Moreover, pairwise comparisons of Φ_{ST} (an F_{ST} analog) indicated highly significant genetic differentiation between all 45 pairs in the 10 populations ($P < 0.0000$) (data not shown).

4. Discussion

The detection of high levels of polymorphism makes ISSR analysis a powerful tool for assessing genetic diversity in *H. littoralis*. None of the individuals was genetically identical based on the ISSRs, indicating that the level of resolution in our study was sufficient to distinguish all genotypes.

Accurate estimates of genetic diversity are useful for optimizing sampling strategies and for conserving and managing the genetic diversity of trees (Hamrick and Godt, 1996). In the present study, all three genetic diversity indices ($P = 93.1\%$, He $= 0.236$, and $I = 0.365$) revealed that genetic diversity of *H. littoralis* is high. This result corresponds to earlier findings that tropical trees tend to present high levels of genetic diversity (Hamrick and Loveless, 1989). However, our estimate is substantially higher than that reported by Das et al. (2001). They used 10 RAPD primers on five plants of *H. littoralis* from Assam, India, and identified 69 loci, 42% of which were polymorphic. Though reduced samples and less sensitive marker system, RAPD, may account for the low level of genetic diversity observed in Das' study, we can not compare the resolution of these two studies due to different localities sampled.

In contrast to the high genetic diversity observed in *H. littoralis*, genetic diversity of two other mangrove species was extraordinarily low. Based on ISSRs, Ge and Sun (2001) examined genetic diversity of *C. tagal* from Thailand and China, and found that the percentage of polymorphic loci (P) and mean expected heterozygosity (He) were 9.0% and 0.016, respectively. ISSR diversity for *A. corniculatum* also was very low at the species level ($P = 16.2\%$, He $= 0.039$) (Ge and Sun, 1999). Lakshmi et al. (1997) suggested that different mangrove species were likely to display varying degrees of polymorphism depending on their edaphic preferences and adaptations.

We found genetic diversity within Australian populations to be higher than in Chinese populations. This outcome may be due to ecogeographic variation since these Australian populations are located at a lower latitude and more tropical region. Fahima et al. (2002) reported that microsatellite polymorphisms in natural populations of wild emmer wheat were best explained by variation of altitude and mean temperature in August. In this study, we observed that intra-population diversity of *H. littoralis* decreased inversely with latitude. However, due to the limited number of sampling sites in this study, further research is needed to determine whether latitude can influence the genetic diversity of this species directly or indirectly, for instance through temperature effects. Another interpretation for the higher genetic diversity observed in Australian populations may be related to the evolutionary history of this species. *H. littroralis* extends from east Africa and Madagascar to the Pacific and reaches its northern limit in Hong Kong (Tomlinson, 1986). Therefore, populations in China, especially those in Yanzao (adjacent to Hong Kong) are marginal populations of this species. It has been well documented that marginal populations are often less variable than populations within the primary range (Blows and Hoffmann, 1993).

A high G_{ST} value (0.426) indicated pronounced genetic differentiation among the 10 populations studied. More than 40% of the genetic variation in our sample can be attributed to variation among populations. Similar results were obtained for other mangrove trees. The estimate of G_{ST} was 0.529 among populations of *C. tagal* in Thailand and China using ISSRs (Ge and Sun, 2001). A RAPD analysis revealed approximately 40% genetic diversity for *Bruguiera sexangula* and 30% for *Bruguiera gymnorhiza* among populations in Sri Lanka (Abeysinghe et al., 2000). In addition, an SSR assessment of population-level genetic variation throughout the worldwide range of *Avicennia marina*, a pioneer mangrove, also indicated that 40% of the total variation represented inter-population differences (Maguire et al., 2002).

AMOVA revealed that differentiation between Chinese and Australian populations accounted for 41.7% of the total genetic variance. This differentiation could be explained by the geographical isolation between China and Australia as indicated by the significant correlation between genetic differentiation and geographic distance among all 10 populations ($r^2 = 0.956$, $P = 0.004$). Gene flow may be weakened across the geographic distance between Chinese and Australian populations, which may explain the observed pronounced differentiation. This effect is reflected in the much lower gene flow found among all 10 populations studied ($N_m = 0.336$) compared to levels within Chinese populations ($N_m = 0.800$) or Australian populations ($N_m = 1.923$). Floating seeds of *H. littoralis* along the Daintree River may contribute to the high level of gene flow observed among Australian populations because those populations are connected hydrologically.

The differentiation observed among both Chinese and Australian populations did not significantly correspond to geographic distance. This result was especially apparent in the Chinese populations. Although populations clustered according to locality, Yanzao populations were genetically closer to Qinglan populations (∼573 km) than to the geographically closer populations in Xiangkeng (∼56 km). In addition, AMOVA revealed that there was significant variance introduced by habitat-correlated genetic differences (5.3%, $P < 0.001$), in addition to considerable geographical-effected genetic variance (22.5%, $P < 0.001$). Therefore, we suggest that besides the effects of gene flow and genetic drifts, local ecological conditions (temperature, water availability, type of sediment etc.) also played an important role in the observed genetic structure of *H. littoralis*. Recently, Bockelmann et al. (2003) used microsatellites to distinguish the effects of habitat differentiation and isolation-by-distance in a salt-marsh plant *Elymus athericus*. They found marked habitat adaptation (AMOVA, 14%) on a small-scale compared with isolation-by-distance (AMOVA, 8.9%) on larger scales. Ecogenic adaptation has also been reported for some widespread mangrove species such as *A. marina* and in the genus *Rhizophora* based on both biochemical and genetic evidences (Rafii et al., 1999; Dodd et al., 1999). In the present study, fluctuating salinity, alternating aerobic, and anaerobic conditions, periodically wet and dry soils, and unstable and shifting substrates in marine environments may influence genetic differentiation between mangroves and woodland populations of *H. littoralis* at a local scale. The genetic habitat adaptations to marine and terrestrial environments may also be a potential barrier to gene flow as reflected in Australian populations. Population DR-1 in the marine environment differed genetically from the other Australian populations. The two freshwater populations showed closer genetic relationships to each other than to the inland population.

The population structure revealed here in *H. littoralis* has clear conservation and management implications. The strong genetic differentiation among populations observed indicates that not only marine but also terrestrial populations of *H. littoralis* should be included in comprehensive conservation projects. Although loss of individuals or populations in some locations may not cause an immediate loss of genetic diversity, it may result in decreased potential to adapt to environmental changes in the long term. If the goal of rehabilitation and restoration is functional equivalence with native local populations, the donor propagules should be selected from populations that have high genetic similarity to one another. For example, plantings of *H. littoralis* in China should use propagules collected from ecologically similar populations to increase their probability of survival. In contrast, if the sites are to be planted with propagules from donor populations, then sampling strategies should

focus on small collections spread across populations covering several sites on a regional scale to obtain the maximum representation of genetic diversity.

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