

## Genetic structure of mourning cuttlefish (*Sepia plangon* Gray, 1849) in Sydney Harbour, Australia

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### ABSTRACT

Knowledge of behaviours, including sex-biased dispersal and kin-association, provides important insight into the costs and benefits of group-living. Such behaviours can be difficult to observe directly, but have quantifiable genetic signatures that allow us to determine their occurrence within and among groups. The mourning cuttlefish *Sepia plangon* Gray, 1849 is both solitary and found in groups at different locations inside Sydney Harbour. Here we describe the genetic relatedness of individuals from four sampling locations, up to 7 km apart, within Sydney Harbour. We also describe the relatedness of juveniles swimming together in small groups of up to seven individuals and the relatedness of 32 adults found in 16 mating pairs, to evaluate the risk of inbreeding. We estimate relatedness with data from 52 amplified fragment length polymorphism (AFLP) loci. We found no evidence that groups of juveniles consisted of related individuals and therefore suggest that kin do not preferentially associate. Additionally, we found that relatedness between adult pairs did not differ from random. Taken together, these results suggest that the risk of inbreeding in this species is low. No genetic structure was detected among groups of juvenile *S. plangon* across our sampling locations, implying regular dispersal and gene flow among locations separated by distances of up to 7 km. We therefore suggest that group formation in *S. plangon* does not incur the risk of inbreeding or tend indirectly to benefit related individuals sharing the same group.

### INTRODUCTION

The formation of intraspecific groups is common in many taxa (Alexander, 1974; Kearney *et al.*, 2001; Stow *et al.*, 2001; Krause & Ruxton, 2002). Factors that influence the formation of groups vary from benefits, such as greater vigilance, foraging efficiency and mating opportunities (Wilson, 1975; Dobson & Poole, 1998; Lancaster, Wilson & Espinoza, 2006), to costs, including conspicuousness to predators, and increased competition for resources and mates (Krause & Ruxton, 2002). These costs and benefits are further complicated by the relatedness of the individuals in the group, because kin may gain direct fitness advantages from helping each other whereas unrelated individuals may not (Hamilton, 1964). If conditions favour the association of individuals that are no more closely related than expected by chance, groups may persist because each individual receives benefits of reciprocity or mutualism (Trivers, 1971; Clutton-Brock, 2009). If a group consists of closely related individuals, its formation may be driven by kin selection where grouping behaviour enhances inclusive fitness (Hamilton, 1964; Pamillo, 1989).

While many benefits of group-living have been identified, several costs must be mitigated for grouping behaviour to persist. Grouping by kin may elevate the risk of inbreeding, if

precopulatory inbreeding avoidance mechanisms are lacking (Jansson, Uller & Olsson, 2005; Billing *et al.*, 2012; Tan *et al.*, 2012). Sex-biased dispersal can reduce the chances of inbreeding (Moore & Ali, 1984; Perrin & Mazalov, 1999; Lehmann & Perrin, 2003), but in group-living species with low levels of dispersal, precopulatory mechanisms for inbreeding avoidance are commonly reported (Blaustein & Waldman, 1992; Stow & Sunnucks, 2004; Liboreau, Zimmer & Rivault, 2007). As population size decreases, these mechanisms become less effective at mediating inbreeding risk due to the increased proportion of related individuals. Taking a genetic approach, it is possible to disentangle how relatedness might influence grouping behaviour from an evolutionary viewpoint.

To avoid the potential costs associated with living in a group, individuals may associate temporarily in groups that form for discrete periods of time. The squid *Sepioteuthis sepioidea* forms temporary shoals during the day potentially to increase vigilance against predators, but disperses to hunt at night (Moynihan & Rodaniche, 1982). Groups may dissolve permanently when individuals move away from their natal areas as juveniles to become solitary adults (Jakob, 1991; Molvar & Bowyer, 1994; Lubin, Henschel & Baker, 2001; Bilde *et al.*, 2007). In brook trout (*Salvelinus fontinalis*), males permanently disperse from the natal

ground to reduce competition with male conspecifics (Hutchings & Gerber, 2002). Both temporary and permanent dispersal can reduce competition for resources and mates, and minimize inbreeding by separating siblings before reproduction, especially if dispersal is sex-biased (Greenwood, 1980; Pusey, 1987; Pusey & Wolf, 1996).

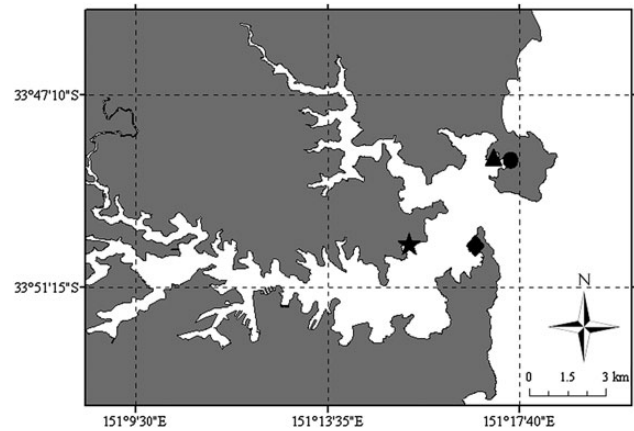
Cephalopods exhibit a spectrum of group behaviours that vary from solitary octopus species to shoaling in some squid (Hanlon & Messenger, 1996; Boal, 2006). Most cuttlefish lead primarily solitary lives, with groupings or temporary pairings only present during breeding seasons (Boal, 2006). A remarkable display of grouping behaviour is that of the hundreds of giant cuttlefish (*Sepia apama*) that form annually in the Spencer Gulf, South Australia, for mating and egg-laying (Hall & Hanlon, 2002). Similarly, the broadclub cuttlefish (*Sepia latimanus*) forms loose aggregations at the peak of its breeding season (Corner & Moore, 1980). Outside these breeding seasons, however, both species are solitary.

The mourning cuttlefish *Sepia plangon* Gray, 1849 is unusual among cuttlefish as its individuals associate both during and outside the mating season (McBride, 2005). In *S. plangon* there is a seasonal shift in both group size and composition, with adults pairing during the breeding season (June–November) and larger groups ( $n > 6$ ) of immature cuttlefish forming in the post-spawning season (McBride, 2011). Behavioural investigations have shown that the pairs of mature cuttlefish are likely to be mating associations (McBride, 2011); however, the function of the juvenile groupings remains unknown. *Sepia plangon* is abundant in sheltered bays along the east coast of Australia and is common in Sydney Harbour (Norman, 2000). Our aims were to use genetic methods to assess (1) relatedness of juvenile cuttlefish within groups to test the relevance of kin selection to this system, (2) relatedness between mating pairs to evaluate the presence of disassortative mating with respect to relatedness and (3) relatedness within and among four sampling locations in Sydney Harbour to assess the risk of inbreeding among individuals postdispersal.

## MATERIAL AND METHODS

A total of 186 tissue samples were collected from *Sepia plangon* at Parsley Bay ( $n = 103$ ), Chowder Bay ( $n = 37$ ), Manly Cove ( $n = 30$ ) and Little Manly Cove ( $n = 16$ ), located in Sydney Harbour, New South Wales, Australia (Fig. 1). The sampling locations were chosen based on the consistently large numbers of cuttlefish found at each site and with no a priori expectations of population structure or lack thereof. Samples were collected between August 2007 and February 2009 at Parsley Bay and between February 2009 and May 2010 at all other sites. For the purposes of this paper, a group consists of two or more animals, with individuals occurring less than 2.5 m away from their nearest neighbour; a pair was defined as two cuttlefish of opposite sexes swimming within 1 m of each other (McBride, 2005). Among the 186 cuttlefish collected, 32 were pairs of adult cuttlefish (16 pairs), of which two pairs were from Chowder Bay and the remaining 14 pairs from Parsley Bay. Forty-six of the cuttlefish collected were found in 10 groups: five from Manly Cove, collected in February 2009 (one group of four, one group of seven) and in March 2009 (one group of six, two groups of four); two from Little Manly Cove collected in May 2010 (one group of three, one group of five) and three from Chowder Bay collected in May 2010 (two groups of four and one group of five animals). Tissue samples from a further five cuttlefish were collected from Moreton Bay, Queensland, Australia (>750 km from Sydney Harbour), between February 2009 and May 2010 as a control measure for population structure.

Cuttlefish were captured by SCUBA diving, using a hand net (mesh size 1 cm<sup>2</sup>) and tissue samples were taken from either the ventral mantle (if whole animal was collected) or arm tip



**Figure 1.** Location of male, female and juvenile *Sepia plangon* collected at four study sites with Sydney Harbour: Manly Cove (triangle), Little Manly Cove (circle), Chowder Bay (star) and Parsley Bay (diamond).

(sample collected *in situ*). Where groups were detected, all members of each group were collected, with several divers targeting separate individuals within each group. Individuals of unknown grouping were also collected. External sex identification and verification of species were based on a comprehensive study of the reproductive biology of *S. plangon* from 2007 to 2010 (McBride, 2011). As the suckers on the hectocotylus are only slightly smaller than those on the normal arms, dorsal patterning was used to indicate sex. Adult males were identified by the presence of transversal dorsal stripes (intensified during mating season) and a mottled colouration in the females (Norman, 2000). Juvenile cuttlefish were less than 50 mm dorsal mantle length (DML) for males, and less than 60 mm DML for females. Lack of gonadal tissue was confirmed by dissection, verifying their nonreproductive status. Upon collection each sample was placed in 70% ethanol and stored at  $-20^{\circ}\text{C}$ .

### DNA extraction and amplification

Total DNA was extracted and purified from all 186 individual tissue samples using Qiagen spin columns. We conducted amplified fragment length polymorphism (AFLP) assays using a protocol that was slightly modified from that described by Vos *et al.* (1995). Digestion of total DNA (200–400 ng) was performed in a 40  $\mu\text{l}$  reaction with 5 U of *EcoRI* and 5 U of *MseI* enzymes (New England Biolabs) for 1 h at  $37^{\circ}\text{C}$ . Adapters were ligated in a 10  $\mu\text{l}$  reaction with 5 pmol of the *EcoRI* adapter, 50 pmol of *MseI* adapter, 1 Weiss Unit of T4 DNA ligase and  $1 \times$  T4 ligase buffer (adapter sequences from Zenger *et al.*, 2007). Adapters were added to the 40  $\mu\text{l}$  total DNA digest and incubated at  $37^{\circ}\text{C}$  overnight. Fragments were amplified in two steps, a preamplification PCR and a selective PCR with primers containing three or four selective nucleotides. Preamplification PCR was a 20  $\mu\text{l}$  reaction containing 1.5 mM  $\text{MgCl}_2$ , 0.2 mM of each dNTP,  $1 \times$  *Taq* polymerase buffer, 1 U *Taq* polymerase (Qiagen) and 75  $\mu\text{g}$  of each primer *EcoRI* (5'GACTGCGTACCAATTCA3') and *MseI* (5'GATGAGTCCTGAGTAAC3'). The preamplification PCR cycling profile was:  $94^{\circ}\text{C}$  for 2 min, 30 cycles at  $94^{\circ}\text{C}$  for 30 s,  $56^{\circ}\text{C}$  for 1 min,  $72^{\circ}\text{C}$  for 1 min and  $72^{\circ}\text{C}$  for 4 min. For the selective PCR step, a 1:10 dilution of the preamplification PCR amplicons was obtained for template. The selective PCR was a 20  $\mu\text{l}$  reaction: 3  $\mu\text{l}$  of 1:10 preamplification PCR product, 1.5 mM  $\text{MgCl}_2$ , 0.2 mM each dNTPs,  $1 \times$  *Taq* buffer, 1 U *Taq* polymerase, 5 ng of fluoro-labelled 3+ *EcoRI* selective primers (PET, FAM, VIC or NED; Applied Biosystems) and 30 ng of 4+ *MseI* selective primers. The selective

PCR profile was: 94 °C for 30 s, 65–58 °C (touchdown—temperature drops 1 °C per cycle for the first 7 cycles) for 30 s, 72 °C for 1 min, then 30 cycles at 58 °C annealing temperature. We used a total of five primer combinations, these were: (1) *MseI* + CTGC with *EcoRI* + AGT, (2) *EcoRI* + ATC, (3) *EcoRI* + AAC, (4) *MseI* + CAAC with *EcoRI* + ATC, (5) *EcoRI* + AAC. Samples were electrophoresed on an ABI3130 (Applied Biosystems) and peaks were scored using Peak Scanner software (v. 1.0, Applied Biosystems).

### Data analysis

For the AFLPs, the number of fragments scored per sampling region, pairwise  $F_{ST}$  and pairwise relatedness were calculated using AFLP-SURV v. 1.0 (Vekemans, 2002). Allele frequencies were calculated using a Bayesian method with a nonuniform expectation of allele frequencies and  $F_{ST}$  between sampling locations was calculated following Reynolds, Weir & Cockerham (1983). Pairwise relatedness values between individuals were generated following Lynch & Milligan (1994). An analysis of molecular variation (AMOVA) was carried using GENALEX v. 6.0 (Peakall & Smouse, 2006). To estimate genetic structure across the population we also generated Nei's unbiased genetic distance D (DeGiorgio & Rosenberg, 2009) and conducted a principle coordinates analysis.

To gauge the level of migration required to generate the  $F_{ST}$  values we observed, we ran simulations using EASYPOP v. 2.0.1 (Balloux, 2000). These simulations consisted of 1000 generations, two sexes, random mating, an island model, 52 loci, free recombination between loci, 0.0001 mutation rate, equal probability mutation model (Kam), two possible allelic states, maximal variation in the initial population and an equal sex ratio. We ran simulations for three migration levels, where 50, 5 and 1% of individuals migrated at each generation.

Pairwise relatedness calculated using AFLP-SURV v. 1.0 was used to evaluate mean relatedness within groups of individuals and mean relatedness at each sampling location. Relatedness was calculated from allele frequencies obtained from all sampled individuals, with the average relatedness set at zero and individual pairwise relatedness ranging from  $-1.0$  to  $+1.0$ . If gene flow is nonrandom among locations, the prediction is that mean relatedness within locations will be elevated. To evaluate whether mean relatedness within sampling groups was higher than a random distribution, the 'Pop Mean' function of GenAIEx v. 6 (Peakall & Smouse, 2006) was used to generate 95% confidence intervals around average relatedness for each location and around the random distribution (for which the mean = 0). To compare pairwise relatedness values for groups of individuals belonging to different age and sex categories, relatedness was compared between groups by randomization testing of the difference in means. Tests in the SAMP module of RT v. 2.1 (Manly, 1997) with 5000 permutations were applied to pairs of adults to test whether relatedness between breeding pairs differed from random and whether groups of immature *S. plangon* consisted of related individuals.

## RESULTS

### Summary data

The five primer-pair combinations selected for AFLP analysis each displayed optimal complexity reduction and easy-to-score polymorphisms. A total of 52 AFLP loci were identified across the five primer-pair combinations. Each fragment was polymorphic in cuttlefish from all sampling locations. The estimated observed heterozygosity ( $\pm 1$  SE) for pooled samples ( $H_0$ ) was 0.411 ( $\pm 0.044$ ) while the estimated expected heterozygosity ( $H_E$ ) was 0.450. Of the 52

**Table 1.** Genetic distance between our four sampling locations in Sydney Harbour.  $F_{ST}$  values shown in lower half of matrix; significance based on 999 permutations shown in upper half of matrix in bold.

	Manly Cove	Chowder Bay	Little Manly Cove	Parsley Bay
Manly Cove		<b>0.054</b>	<b>0.287</b>	<b>0.127</b>
Chowder Bay	0.015		<b>0.426</b>	<b>0.071</b>
Little Manly Cove	0.004	0.000		<b>0.441</b>
Parsley Bay	0.006	0.008	0.000	

**Table 2.** Genetic distance between the sampling locations in Sydney Harbour following the pooling of samples from Manly and Little Manly Coves.

	Manly Cove	Chowder Bay	Parsley Bay
Manly Cove		<b>0.141</b>	<b>0.236</b>
Chowder Bay	0.007		<b>0.082</b>
Parsley Bay	0.003	0.008	

$F_{ST}$  values are shown in lower half of the matrix; significance based on 999 permutations is shown in the upper half of the matrix in bold.

AFLPs identified, 39 were variable in individuals sampled from Moreton Bay.

### Genetic partitioning among sampling locations in Sydney Harbour

Pairwise  $F_{ST}$  showed that there was very little, if any, allelic partitioning among sampling locations ( $F_{ST}$  range = 0.000–0.015, average  $F_{ST} \pm SE = 0.0055 \pm 0.0002$ , all  $P$  values  $> 0.05$ ; Tables 1 and 2). Nei's D ranged from 0.004 to 0.174 and the first three axes of the principal components analysis explained 15.95, 7.33 and 5.79% of the variation in the sample, with a cumulative total of 29.07%. Given the proximity of the two Manly sites, we ran the  $F_{ST}$  analysis with the Manly sites pooled, which returned similar results (Table 2). While there was some variation in sample sizes at the localities, the consistency of results across localities suggests that this created little bias. AMOVA showed that 99% of the variation was within sampling localities, further demonstrating very little genetic partitioning among locations across Sydney Harbour. It is also clear that our markers had the resolution to detect genetic structure, because substantial differences in allele frequencies were apparent between samples collected in Sydney Harbour and those collected in Moreton Bay, about 700 km away. AMOVA showed that 27% of the variation occurred between samples collected from Sydney and those from Moreton Bay, while in Sydney Harbour 99% of the variation was within sampling localities, demonstrating very little genetic partitioning among the Sydney Harbour locations. This pattern was reflected by the pairwise  $F_{ST}$  values, which were large and significant ( $> 0.170$ ; probability of no structure,  $P < 0.001$ ) between Sydney Harbour locations and Moreton Bay, whereas there was very little partitioning among sampling localities within Sydney Harbour ( $F_{ST}$  range: 0.0016–0.0089).

### Migration estimates by simulation tests

The three simulations run using EASYPOP v. 2.0.1 showed that our observed genetic structure ( $F_{ST} = 0.0055$ ) was lower than the simulated  $F_{ST}$  obtained when 5% of individuals migrate at each generation, but greater than the genetic structure obtained

when 50% of individuals migrate (average  $F_{ST} \pm SE$  at 50% migration =  $0.0017 \pm 0.0002$ ; 5% migration =  $0.034 \pm 0.0007$ ; 1% migration =  $0.181 \pm 0.001$ ). These data show that >5% of cuttlefish migrate between each location at each generation.

#### Patterns of relatedness within Sydney Harbour

The mean relatedness among individuals sampled within each location in Sydney Harbour did not differ significantly from random (i.e. relatedness = 0), and none of the sampling locations differed significantly in their level of relatedness from one another (Fig. 2). These results suggest that the individuals are part of one population.

#### Relatedness of juveniles in groups

The observed range of pairwise relatedness values within the 10 juvenile groupings (of 3–7 individuals) was  $-1$  to  $0.65$ . The average pairwise relatedness ( $\pm 1$  SE) among juvenile groupings was  $0.04$  ( $\pm 0.04$ ). Overall, groupings did not show a significantly higher level of relatedness among individuals than would be expected by chance ( $P = 0.31$ ).

#### Relatedness between pairs of opposite sex

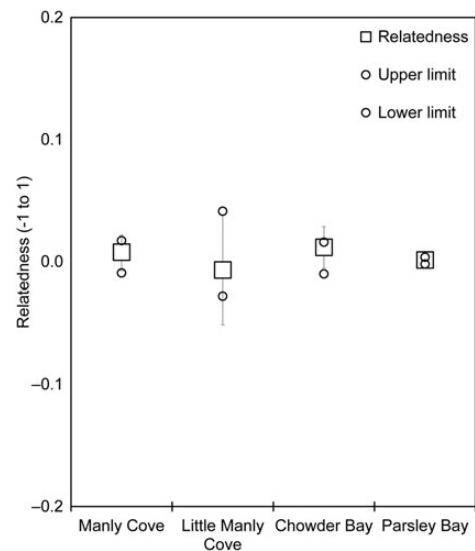
Pairwise relatedness for the 16 pairs of opposite sex ranged from  $-1.0$  to  $0.63$ , with an average ( $\pm 1$  SE) pairwise relatedness of  $0.06$  ( $\pm 0.08$ ). The mean pairwise relatedness for pairs of opposite sex did not differ significantly from chance ( $P = 0.51$ ).

## DISCUSSION

We found no evidence for genetic structuring among mourning cuttlefish, *Sepia plangon*, in our four locations in Sydney Harbour. Our simulations showed that the genetic structure we observed could result from a migration rate that is greater than 5% of individuals per generation. We found that juvenile individuals within groups were no more closely related to each other than would be expected by chance; therefore, either kin do not preferentially associate in the mourning cuttlefish or clutches are sired by many males (Squires *et al.*, 2012). Also, breeding pairs of mourning cuttlefish were no more closely related than expected by chance, consistent with the low relatedness observed among individuals in the juvenile groups.

The lack of genetic structure in the mourning cuttlefish is surprising, though the lack of genetic distinction between Manly Cove and Little Manly Cove may not be, given their separation by *c.* 1 km (McBride, 2011). Adult *S. plangon* have a home range of less than 800 m<sup>2</sup> for most individuals, but show short to medium term site fidelity (McBride, 2011). Our results show gene flow in *S. plangon* across up to 7 km. This contrasts with the strong population structure found in *S. officinalis* across 25 km around the Iberian Peninsula (Pérez-Losada *et al.*, 2002). Our evidence for genetic mixing could possibly be explained by recent separation and the retention of ancestral alleles ('Slatkin's Paradox'; Marko & Hart, 2011), but differences in pairwise relatedness are generated over much shorter time scales than are differences in allele frequency (Stow *et al.*, 2001). Therefore the lack of relatedness structure within the harbour may indeed imply gene flow (and potentially dispersal) over distances of up to 7 km.

As evidenced by our study, *S. plangon* juveniles form unrelated groups. These groups may form via a mechanism that promotes random mixing, such as passive juvenile dispersal (Payne, Semmens & Gillanders, 2011). Some cephalopod species have planktonic juveniles that undergo passive dispersal (Boletzky, 2003; Boyle & Rodhouse, 2008). However, Nixon & Mangold (1998) suggested that juvenile *S. officinalis* are nondispersive,



**Figure 2.** Mean pairwise relatedness of all samples within four populations in Sydney Harbour. Upper and lower limits represent 95% confidence intervals surrounding zero (calculated by bootstrapping).

because they hatch with a body form similar in morphology to adults which allows controlled swimming and burying behaviour. As *S. plangon* hatch at *c.* 1 cm in total body length and are very similar to adults (McBride, 2011), their dispersal may be similar to that of *S. officinalis*. Thus it is unclear whether passive dispersal is an important mechanism of gene flow in *S. plangon*.

Because members of groups were not more closely related than expected by chance, factors that promote grouping in this species are unlikely to be linked to kin selection. While evidence for non-kin group benefits (Clutton-Brock, 2009) is absent for this species, there is some evidence of intraspecific communication in *S. plangon* (Brown, Garwood & Williamson, 2012). If *S. plangon* groups are cooperative, their formation may be driven by mutualistic mechanisms such as resource dependence or vigilance. This idea could be tested by manipulative experiments that examine responses to predation risk in the presence or absence of conspecifics.

Alternatively, the groups of juvenile *S. plangon* could be genetically diverse if each is a single clutch sired by many males. Populations of *S. plangon* are male biased (McBride, 2011) and males deceive rival males during courtship (Brown *et al.*, 2012), a behaviour associated with strong sexual competition and polygamy. Offspring of polygamous mating systems are typically genetically diverse and this may relax selection on kin recognition (Pusey & Wolf, 1996; Tregenza & Wedell, 2002). Polygamy has been documented in several species of cephalopod (Shaw & Sauer, 2004; Rey-Méndez, 2011; Squires *et al.*, 2012). For example, Hall & Hanlon (2002) found that both male and female *S. apama* often mate with multiple mates. Additionally, female *S. apama* often deposit clutches of eggs sired by multiple males (Naud *et al.*, 2004). We suspect that *S. plangon* may also be polygamous, because both sexes have been observed to leave their initial partner after mating and to form another pair (McBride, unpublished field observation). Pairs were no more closely related than expected by chance, possibly owing to dispersal as discussed above. If the risk of encountering a related mate is low, *S. plangon* has no need to detect and avoid kin as breeding partners (Pusey & Wolf, 1996). Further data are required to determine whether *S. plangon* adults actively choose genetically distinct mates (e.g. as in Lihoreau *et al.*, 2007) or whether strong genetic mixing has relaxed the need to detect

genetic similarities in potential mates. Nonetheless, these first genetic data on groups of *S. plangon* demonstrate that there is no biological evidence for kin selection to explain the unusual grouping behaviour in this species and that inbreeding is not likely to be common.

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