Evidence of *in situ* competition between fungi in freshwater

Sally C. Fryar[1*], Webber Booth[2], Jonathan Davies[2], I. John Hodgkiss[1], and Kevin D. Hyde[1]

[1]Centre for Research in Fungal Diversity, Department of Ecology & Biodiversity, The University of Hong Kong, Pokfulam Road, Hong Kong
[2]School of Biology, Universiti Brunei Darussalam, Brunei Darussalam

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Five species of fungi, isolated from submerged wood collected from a Brunei peat swamp forest, were tested for their competitive ability. *Annulatascus velatisporus*, *Beverwykella pulmonaria*, *Dactylella* sp., *Monodictys pelagica* and *Pleurophragmium* sp. were paired in all possible combinations on wood and agar in the laboratory. In addition, autoclaved wood blocks were inoculated with these isolates and placed back into water in the peat swamp forest. On agar, there was a competitive hierarchy of *Dactylella* sp. > *Pleurophragmium* sp. > *A. velatisporus* > *B. pulmonaria* > *M. pelagica*. In the inoculated blocks exposed in the peat swamp forest however, *M. pelagica*, *A. velatisporus* and *B. pulmonaria* were the only species to significantly reduce the colonisation of other fungi, showing a strong disparity between field and laboratory results. Laboratory studies on wood revealed that *A. velatisporus* reduced the reproductive capability of other fungi. This study demonstrates the influence of interspecific competition in fungal colonisation of submerged wood.

Key words: aquatic fungi, competition, *in situ*, natural substrate, wood.

Introduction

In heterotrophic aquatic ecosystems, where autochthonous production is low, such as in forest streams, degradation of allochthonous litter is critical (Tank and Webster, 1998). Degradation of leaves in such systems involves a diverse assemblage of bacteria, fungi and invertebrates (Gessner, 1999; Gessner *et al*., 2003). There has been some research on determining which organisms are involved in wood degradation in freshwater systems (Tank and Webster, 1998) that indicates that fungi play a key role (Tank and Winterbourn, 1995, 1996; Abdel-Raheem and Shearer, 2002).

Biodiversity studies on fungi that colonise decaying wood in freshwater have revealed a diverse assemblage of ascomycetes and anamorphic fungi

*Corresponding author: Sally C. Fryar; e-mail: scfryar@bigpond.com; present address: 23 Onyx Rd, Artarmon NSW 2064, Australia

(Tsui *et al*., 2000; Cai *et al*., 2002, 2003; Ho *et al*., 2001, 2002; Sivichai *et al*., 2002; Fryar *et al*., 2004a, b). Different groups of fungi are found on different substrates within fresh water (Shearer, 1993; Goh, 1997; Cai *et al*., 2003) and while the ecology of fungi on leaves in freshwater has been relatively well-studied (e.g. Barlocher, 1980, 1982; Suberkropp and Chauvet, 1995; Suberkropp, 1997), less is known about the ecology of the fungi that colonise and degrade wood in freshwater.

Competitive interactions between fungi have the potential to influence the colonisation of substrates, community composition and organization (e.g. Brame and Flood, 1983; Holmer and Stenlid, 1996; Schmidt, 1999; Baar and Stanton, 2000; Fryar *et al*., 2001). There have been numerous studies demonstrating strong competitive interactions between fungi, however the majority of these studies have been undertaken in a controlled laboratory situation either on natural (e.g. Hulme and Shields, 1972; Webber and Hedger, 1986; Wardle *et al*., 1993; Holmer and Stenlid, 1996, 1997) or artificial substrates (e.g. Asthana and Shearer, 1990; Tsuneda and Thorn, 1994; Baar and Stanton, 2000; Fryar *et al*., 2002). Although such studies are able to elucidate many facets of fungal competition that cannot be studied in the field and are useful for observing the behaviour of fungi, it is essential to attempt to link laboratory results with the natural situation (Shearer, 1995). While some field based and *in vitro* competition studies have resulted in similar conclusions (e.g. Hulme and Shields, 1970; Panebianco *et al*., 2002), many others are contradictory (e.g. Magan and Lacey, 1984; Webber and Hedger, 1986; Fryar *et al*., 2001).

A well-used method for detecting the influence of competition *in situ* is to manipulate the abundance of one or more hypothetically competing species (Shearer, 1995). Such manipulations may be removals, introductions, or both (Schoener, 1983). There have been few field experiments on interspecific competition between fungi. Shearer and Bartolata (1990) demonstrated interspecific competition in a stream by pre-colonising birch wood sticks in the laboratory with individual species differing in competitive ability in paired culture on agar, before exposing them in the stream. Fryar *et al*. (2001) also demonstrated the influence of competition on wood, however, the effect of competition was only weak. Panebianco *et al*. (2002) found that colonisation of fungi in wood blocks in Langstone Harbor was inhibited by the presence of pre-inoculated marine fungi.

The importance of competition in determining the distribution and abundance of fungi in submerged wood is questionable. While, strong competitive interactions have been reported from *in vitro* studies of freshwater fungi from wood (Asthana and Shearer, 1990; Yuen *et al*., 1999), in a field

based study only weak competition was detected (Fryar *et al*., 2001). It is the intention in this study to investigate the importance of competition in the fungal colonisation of submerged wood.

Fifteen species of ascomycetes and asexual fungi were found on wood collected from water in a peat swamp forest in Brunei (Fryar *et al*., 2004a). The present study was conducted at this same site in order to determine the strength of competition between freshwater fungi in the field. It was hypothesized that when wood blocks were inoculated with strongly competitive fungi and introduced into the natural environment, fewer colonising species would be found as compared to control (uninoculated) blocks.

Experimental procedures

Study site

The study site is a shallow peat swamp forest dominated by *Campnosperma coriacea*, *Lophopetalum mutlinervium* and *Syzygium* sp. The area usually has pools of stagnant water that drain into the Sungai Kelakas, a tributary of the Tutong River in Brunei. Occasionally, during periods of high rainfall, the area is inundated with fresh water to a depth of approximately 1m.

Cultures

Wood samples were collected from the Tutong river, Brunei and one of its tributaries, the Sungai Kelakas (Fryar *et al*., 2004a). All samples were collected at the same site, taken to the laboratory, incubated for up to 1 month and examined for fungal fruiting bodies under a dissecting microscope (up to 100×). Fungal species were identified and cultured (see Fryar *et al*., 2004a for methodology). Five isolates were randomly selected for this study: *Annulatascus velatisporus* (HKU(M) 15537); *Beverwykella pulmonaria* (HKU(M) 15544); *Dactylella* sp. (HKU(M) 15637); *Monodictys pelagica* (HKU(M) 15623); and *Pleurophragmium* sp. (HKU(M) 15613).

Species studied

Annulatascus velatisporus is an ascomycete commonly found on submerged wood in freshwater habitats and forms black immersed or semi-immersed ascomata, up to 450 µm high and 260-410 µm wide (Hyde, 1992; Ho and Hyde, 2000).

Beverwykella pulmonaria is an aeroaquatic hyphomycete only recently identified from Borneo (Fryar *et al*., 2004a). Aero-aquatic fungi typically form

in stagnant water where near-anaerobic conditions prevail. These fungi are capable of growing on submerged substrates, but sporulate only when the substrate is exposed to the air. They produce conidia or propagules which trap air, making them effective flotation devices (Goos, 1992).

Monodictys pelagica is a cosmopolitan species of hyphomycete (Jones, 1993) which forms relatively large black conidia approximately 15-38 × 12-35 µm (Mouzouras and Jones, 1985). It is commonly found on wood in marine and brackish water habitats (e.g., Hyde, 1989; Maria and Sridhar, 2003; Fryar *et al*., 2004a) and less commonly in freshwater (Fryar *et al*., 2004a) where the isolate used in this study originated. This fungus has been shown to cause significant weight loss in wood (Mouzouras, 1986) and is the anamorph of *Nereiospora cristata* (Mouzouras and Jones, 1985).

The final two species *Pleurophragmium* sp. and a *Dactylella* sp. are asexual fungi with hyaline hyphae, conidiophores and conidia, found in fresh and brackish water (Tubaki, 1965; Iqbal, 1992; Fryar *et al*., 2004a, b).

In vitro competition studies on agar

The five isolates (Table 1) were paired in all possible combinations on potato dextrose agar (PDA Difco). Inocula were prepared by taking a 5mm diam. agar plug from the growing edge of a colony using the wide end of a sterile Pasteur pipette. Inocula of different species were placed with the centre of each inoculum disc 2 cm apart in the centre of the plate. Control plates were inoculated twice with the same strain. In addition, each strain was inoculated individually onto agar plates to estimate growth rates. There were five replicates of each treatment and control. Mycelial growth of each colony on each agar plate was recorded weekly until there were no further changes in the interactions (after 12 weeks). At the end of this period the area covered by each colony was traced onto the lid of the Petri dish using a permanent marker pen. The lid was then photocopied. The image was captured and the area of each colony was estimated using Leica Quantinet 500+.

The area covered by each strain when paired with other strains was compared using a one-way ANOVA followed by Bonferoni post-hoc tests.

In vitro competition studies on wood blocks

Isolates were also inoculated onto 1 × 1 × 5 cm^3 of untreated *Populus* sp. wood blocks. Before inoculation, blocks were soaked in sterile distilled water for 2 hours and then autoclaved. Inoculation was via 5mm diam. agar plugs of

Table 1. Results of pairwise interactions on agar. +, - 0 = area of target species was greater than, less than or the same as in the control treatments respectively based on ANOVA followed by Bonferoni post-hoc tests ($p<0.05$).

Competitor	**Target species**				
	***Dactylella* sp.**	***Pleurophrag* sp.**	***A. velatisporus***	***B. pulmonaria***	***M. pelagica***
Dactylella sp.		-	-	-	-
Pleurophrag sp.	+		-	-	0
A. velatisporus	+	+		-	-
B. pulmonaria	+	+	+		0
M. pelagica	+	0	+	0	

actively growing mycelia. Inocula were placed on one end of a wood block. Wood blocks were then placed onto an agar plate containing 25 mL of set agar made with sterile distilled water. After two weeks all blocks had mycelium covering the outside. For each species, a wood block inoculated with that species was placed end to end on freshwater agar plates with a wood block inoculated with one of the other species. The process was repeated to ensure all possible combinations of inoculated wood blocks were combined. Blocks were placed so that the inoculation plugs did not touch. Two wood blocks containing the same species were combined as controls. The plates were then sealed with Parafilm and incubated at 27°C for 12 months. Hyphae and fruit bodies on the surface of the wood were observed each month.

In situ competition

Wood blocks, the same as those used in the laboratory wood competition study described above, had holes drilled at each end to allow the blocks to be tethered in the field. They were then autoclaved and inoculated in the same way as in the laboratory wood competition study. These blocks were placed into five stagnant pools and tethered to pneumatophores and trees into five pools. Wood blocks were numbered with stainless steel discs and randomly distributed amongst the five pools. After 12 months, the wood blocks were removed from the water and taken to the laboratory. They were observed under a dissecting microscope immediately after removal from the water and after incubation at 27°C for three months. Any sporulating structures observed were identified and recorded.

Results

In vitro competition studies on agar

On agar plates the competitive hierarchy of the five species was *Dactylella* sp. > *Pleurophragmium* sp. > *Annulatascus velatisporus* >

Beverwykella pulmonaria > *Monodictys pelagica* (Table 1). *Dactylella* sp. overgrew all other species ($F_{4,20}$ = 397.4, $p<0.001$, Fig. 1a). Generally, the losing competitor was reduced to occupying only the initial inoculation plug. However, *Pleurophragmium* sp. occupied an area approximately 1.5 cm diam. *Pleurophragmium* sp. was overgrown by *Dactylella* sp. but overgrew both *A. velatisporus* and *B. pulmonaria*, and shared the resource equally with *M. pelagica* ($F_{4,18}$ = 66.5, $p<0.001$, Fig. 1b). It is interesting that both *B. pulmonaria* and *M. pelagica* occupied a greater area when in competition with *Pleurophragmium* sp. than *A. velatisporus*, even though *A. velatisporus* overgrew both *B. pulmonaria* and *M. pelagica*. *Annulatascus velatisporus* was overgrown by *Dactylella* sp. and *Pleurophragmium* sp., but overgrew both *B. pulmonaria* and *M. pelagica* ($F_{4,19}$ = 2663.6, $p<0.001$, Fig. 1c). *Beverwykella pulmonaria* was overgrown by all other species except *M. pelagica*, with which it shared the resource equally ($F_{4,18}$ = 47.7, $p<0.001$, Fig. 1d). *Monodictys pelagica* was overgrown by *Dactylella* sp. and *A. velatisporus*, but shared the agar plates equally with *Pleurophragmium* sp. and *B. pulmonaria* ($F_{4,19}$ = 236.2, $p<0.001$, Fig. 1e).

In vitro competition studies on wood blocks

Each of the strains had completely covered the wood blocks after two weeks. *Annulatascus velatisporus* was the only species that did not form reproductive structures on the wood blocks. The presence of *A. velatisporus* on wood blocks was apparent by the presence of dark hyphae characteristic of this species. *Pleurophragmium* sp. reproduced sparsely.

After 12 months, *A. velatisporus* had still not produced fruit bodies on the blocks of wood. *Dactylella* sp. no longer produced conidia, whereas all other species still produced conidia. *Monodictys pelagica* and *B. pulmonaria* still reproduced vigorously, with the wood blocks being thickly covered by conidia.

On the wood blocks paired with different species, *M. pelagica* produced a thick covering of conidia on the wood blocks on which it was inoculated and paired, with the exception of the blocks containing *Annulatascus velatisporus*. On these blocks *M. pelagica* only reproduced sparsely. With the exception of when paired with *A. velatisporus*, *B. pulmonaria* also produced a thick covering of conidia on wood blocks in competition. It did not produce any conidia on the block originally inoculated with *A. velatisporus* and produced conidia only sparsely on the block in which it was originally inoculated. *Pleurophragmium* sp. continued to reproduce on all wood blocks on which it was inoculated and on all the blocks with which it was paired. The only

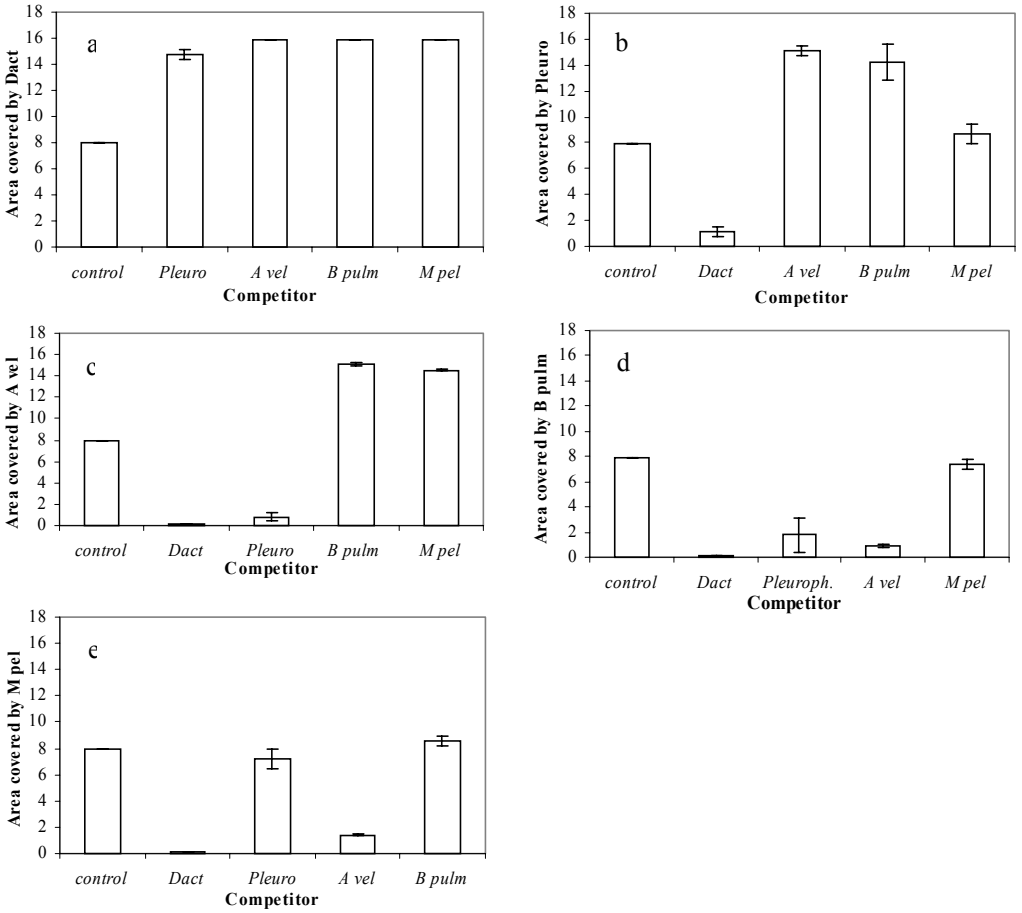

Fig. 1. Graphs showing the area covered by: **a.** *Dactylella* sp. (Dact); **b.** *Pleurophragmium* sp. (Pleuro); **a.** *A. velatisporus* (A vel); **d.** *B. pulmonaria* (B pulm), and; **e.** *M. pelagica* (M pel) when paired with each other species on agar plates.

exception was when *Pleurophragmium* sp. was paired with *A. velatisporus*. In this case, *Pleurophragmium* sp. did not sporulate on either the block on which is it was inoculated or paired.

In situ competition

Pre-inoculated wood blocks had the same mean number of species as the control blocks ($F_{5,96}$ = 0.735, p = 0.599) (Table 2). However, this number includes the pre-inoculated species. Pre-inoculated species were removed from the data, therefore giving the number of new species colonising the wood blocks during exposure *in situ*. Overall, there were less species colonising the

Table 2. Species colonising exposed wood blocks inoculated with different species. B pulmo = *Beverwykella pulmonaria*, M pel = *Monodicyts pelagica*, A vel = *Annulatascus velatisporus*, Pleuro = *Pleurophragmium* sp. and Dact = *Dactylella* sp.

Invading species	**Treatment**					
	Control (n = 11)	**B pulmo (n = 19)**	**M pel (n = 15)**	**A vel (n = 19)**	**Pleuro (n = 19)**	**Dact (n = 15)**
Annulatascus velatisporus	-	2	1	13	1	-
Berkleasmium sp.	-	1	-	-	-	1
Beverwykella pulmonaria	9	17	12	15	17	13
Cancellidium applanatum	8	17	5	8	11	13
Dactylella sp.		-	-	-	1	-
Lasiosphaeria sp.		-	1	-	-	-
Monodictys pelagica		7	8	15	6	8
Monodictys sp.		2	-	-	-	1
Penicillium sp.		-	1	1	-	-
Phaeoisaria clematidis		2	4	1	-	2
Pleurophragmium sp.		-	-	-	-	3
Unidentified hyphomycete	-	2	-	-	1	1
Number of invading sp.		5	8	5	4	7
Mean no. of species	2.6 ± 1.1	2.8 ± 1.4	2.5 ± 0.8	2.3 ± 0.7	2.3 ± 1.0	2.7 ± 0.9
Mean no. of invading sp. per block	2.6 ± 1.1	1.8 ± 1.3	1.4 ± 0.8	1.6 ± 0.8	2.2 ± 0.8	2.7 ± 0.9

pre-inoculated wood blocks when compared with the control blocks ($F_{5,96}$ = 4.35, p = 0.001). *Monodictys pelagica*, *A velatisporus and B. pulmonaria* all reduced the number of colonising species sporulating on the wood blocks on which they were inoculated ($p<0.05$, post-hoc LSD tests).

Five species sporulated on the control wood blocks, including two of the test species (*B. pulmonaria* and *M. pelagica*) (Table 2). *Annulatascus velatisporus* sporulated on 68% of the wood blocks on which it was inoculated. It also colonised several other wood blocks. Only four species colonised the *A. velatisporus* blocks. *Monodictys pelagica* sporulated on all of the wood blocks on which it was inoculated, with only five other species invading these blocks. *Beverwykella pulmonaria* sporulated on most of the wood blocks onto which it was inoculated. However, a large number of other species colonised these blocks, with *Cancellidium applanatum* being the most common. *Pleurophragmium* sp. sporulated only on three of the wood blocks on which it was inoculated, indicating that it was competitively excluded by colonisers. *Cancellidium applanatum* and *B. pulmonaria* were both common on the blocks inoculated with *Pleurophgarmium* sp. and *M. pelagica* sporulated on half of them. *Dactylella* sp. did not sporulate on any of the blocks on which it was inoculated and these blocks were colonised by seven other species. Once again, *C. applanatum* and *B. pulmonaria* were both common on these blocks.

Strong colonisers on all wood blocks were *M. pelagica*, *C. applanatum* and *B. pulmonaria*.

Discussion

Pre-inoculation gave *M. pelagica*, *A. velatisporus* and *B. pulmonaria* a competitive advantage when wood blocks inoculated with these species were placed into water bodies in the peat swamp forest. Competition by these fungi has appeared to affect the subsequent colonisation by other fungi, specifically; competition reduced the number of other species sporulating on the substrate. This is one of only a few studies that have demonstrated the influence of competition on fungal colonisation of substrates (e.g. Shearer and Bartolata, 1990; Fryar *et al*., 2001; Panebianco *et al*., 2002) and is in agreement with these other studies in that pre-colonisation appears to influence subsequent colonisation of fungi.

Pre-colonisation may have influenced the subsequent colonisation by other fungi, or simply reduced the sporulation of other fungi. There are instances in the mycological literature of certain fungal species altering the sporulation of other fungi, but not necessarily altering their mycelial biomass (e.g. Page, 1959; Hutchinson and Cowan, 1972). Methods for observing mycelial occupation of wood may be essential to further clarify the role of fungal competition in structuring assemblages of organisms in wood. Although the observation of fruit bodies on wood is a useful tool for assessing fungal colonisation, there is an underlying assumption that the number of species fruiting on a piece of wood is correlated with the number of species occupying space in the wood (Hyde and Jeewon, 2003).

It is difficult to interpret the results of the laboratory-based wood competition experiment. *Annulatascus velatisporus* appears to have successfully inhibited all other species. Although *A. velatisporus* itself did not produce fruit bodies, it reduced the production of fruit bodies in all other species. *Dactylella* sp. no longer produced fruit bodies, even in the controls, after 12 months. So it is difficult to determine what effect, if any, competition had on this species. *Monodictys pelagica* and *B. pulmonaria* both produced robust resting reproductive structures which could have been produced early in the competition experiment. This would mask any later effect that a competitor may have against these two species. Methods for observing hyphal occupation of space may be essential to understanding competition in wood.

When the field results are compared with the agar plate competition experiments, there is little agreement. The three species that were competitive in the field (*M. pelagica*, *A. velatisporus* and *B. pulmonaria*), were the least competitive in agar plate experiments (*Dactylella* sp. > *Pleurophragmium* sp. > *Annulatascus velatisporus* > *Beverwykella pulmonaria* > *Monodictys pelagica*). The reasons for this are unknown. One possible hypothesis is that

while *M. pelagica*, *A. velatisporus and B. pulmonaria* may be effective in holding already colonised territory, they may not be as good in secondary resource capture. On agar plates, *Dactylella* sp. and *Pleurophragmium* sp. were fast growing. The other species when paired with these would therefore need to capture the resource from them. If *M. pelagica*, *A. velatisporus* and *B. pulmonaria* are not good at secondary resource capture, then this could account for the discrepancy between field results and agar plate results.

Dactylella sp. did not sporulate on wood blocks in the laboratory after 12 months of incubation which is consistent with if it utilises readily available carbon sources rather than the recalcitrant lignocellulose. Most *Dactylella* species are nematode trapping fungi (Lui and Zhang, 2003) and are unlikely to be able to digest lignocellulose (Dong *et al*., 2004). This could explain the disappearance of this species from *in situ* wood blocks and it's lack of competitive ability in the field.

The disparity between competition experiments on agar plates and more natural substrates has previously been observed (e.g. Magan and Lacey, 1984; Webber and Hedger, 1986), but is not well understood. Even fungal competition experiments on different types of agar produce differing results (Whipps, 1987; Wardle and Parkinson, 1992). The current methods used for studying competition in natural substrates however, have inherent problems. Natural substrates need to be sterilized so that other organisms present do not affect the experiment. This is usually carried out by autoclaving, which is known to have an impact on the developing fungal community in wood (Fryar *et al*., 2001).

This *in situ* experiment has demonstrated the influence of competition by pre-inoculated fungi on subsequent sporulation of other fungal species, and possibly colonisation by other species. While *in vitro* competition experiments are useful for understanding mechanisms of competition between fungi, given the often-found disparity between *in vitro* and *in situ* results, it is important to attempt to test the influence of competition in a natural situation.

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