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Co-occurrence of two *Eupristina* species on *Ficus altissima* in Xishuangbanna, SW China

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Abstract
The fig-fig wasp mutualism has long been considered strictly species-specific. However, more and more cases of breakdown in the one-to-one rule are being documented. Here, we report on the co-existence of *Eupristina altissima* and *Eupristina* sp. in the figs of *Ficus altissima* in Xishuangbanna, SW China. Species coexisted on the same tree, even in the same fig. Most crops sampled during a three year survey had both species, but *E. altissima* was more frequent. Brood sizes, when figs were entered by a single foundress, were not significantly different between species. However, our study strongly suggests that *E. altissima* is an active pollinator, while *Eupristina* sp. does not actively pollinate *Ficus altissima*, as only very few seeds were observed in figs only entered by this species. Morphological analysis corroborated this finding: *Eupristina* sp. has reduced pollen pockets and there is no coxal comb, two morphological characters normally linked with active pollination. This is the second report of a non-pollinating agaonid fig wasp, and opens interesting perspectives on the evolution of cheating in fig pollinators.

Keywords: Agaonidae, *Ficus*, pollination mutualism, cheating

1. Introduction

It is now widely accepted that figs and agaonids have strongly related histories (Machado et al., 2001; Rønsted et al., 2005). Formerly, each fig species was thought to be pollinated by one species-specific pollinating wasp: the one-to-one rule. However, exceptions to this one-to-one rule have been long been known, suggesting that the relationship between figs and their pollinators is not always so prescribed (Compton, 1990; Compton and Ware, 1992; Ware and Compton, 1993; Michaloud et al., 1996; Kerdelhué et al., 1997, 1999; Lopez-Vaamonde et al., 2002; Molbo et al., 2003; Su et al., unpublished data; and see Jousselin et al., 2001 for one example of non-agaonid wasp as co-pollinators).

Rasplus (1996) estimated that about 20% of fig-fig pollinator associations broke the one-to-one rule, and described six situations with two co-occurring pollinators. He emphasized the fact that two pollinators can coexist on a same fig species. For example, *Ceratosolen flabellatus* Grandi and *C. silvestrianus* Grandi both actively pollinate *Ficus sur* in Ivory Coast, West Africa (Kerdelhué et al., 1997). While, *C. arabicus* Mayr and *C. galili* Wiebes co-occur on *F. sycomorus* in southern Africa. However, *C. galili* is a "cuckoo", and never carries pollen (Compton et al., 1991).

One of two scenarios can explain the occurrence of more than one species-specific pollinator on a single host fig; there may have been a recent speciation event in the pollinator not concomitant or followed by a speciation event in the *Ficus* species, or the fig may have been colonized by the pollinator of another *Ficus* species that subsequently became extinct (Michaloud et al., 1996; Kerdelhué et al., 1999). Moreover, genetic data has demonstrated that some cryptic species pairs share the same host (Moble et al., 2003). These reveal that colonization events involving wasp multiple symbionts provide a likely scenario for the transition between mutualism and parasitism.

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In India, *Ficus altissima* is pollinated by *Eupristina altissima* Balakrishnan, Joseph & Abdurahiman, 1981. The fig species has also been introduced in the USA, where it is pollinated by *E. altissima* (McKey, 1989; Patel et al., 1993). In Xishuangbanna in the tropical southwest of China, we found *E. altissima* and a second, undescribed *Eupristina* sp. co-occurring on *Ficus altissima* (Gu et al., 2003). The present study focuses on understanding the co-existence of these two species on *F. altissima*, and addresses the following questions: (i) Do both species occur on the same tree at the same time? (ii) Can both species successfully reproduce in the figs of *Ficus altissima*? (iii) Are both species of *Eupristina* effective pollinators?

2. Materials and Methods

Ficus altissima Blume (section *Conosyceae*) is distributed across Asia (Corner, 1965). The hemi epiphytic species can reach very large proportions, over 40 m tall and, through the growth of its aerial roots, occupy a patch of about 300 m^2. The species occurs naturally in tropical forests, but is frequently planted in cities, villages or near temples both as ornamental and as a sacred plant. In Xishuangbanna, *F. altissima* produces figs throughout the year in synchronous crops with asynchrony between trees. The figs are axillary and paired (rarely solitary). Mature figs are subglobose, measure 14.1±1.11 mm in diameter, and contain on average 584.6 ± 93.56 female flowers and 53.3±15.06 male flowers (mean±SD, n=60) (unpublished data).

In Xishuangbanna, a diverse community of fig wasps (25 species) developed within the figs of *F. altissima*. The two agaonid species belong to the genus *Eupristina* Saunders, and are morphologically similar. Nevertheless, females of each species differ in several characters and can be easily separated (Table 1). Up until now, we have not been able to find characters to separate the males.

Table 1. Diagnostic characters used to distinguish the females of *Eupristina altissima* and *Eupristina* sp. that co-occur on *Ficus altissima* in Xishuangbanna, SW China.

Species	Body colouration	Antennae shape
E. altissima	Ventral side of thorax grey	Antenna with 7 funicular segments, clava is unisegmented
Eupristina sp.	Ventral side of thorax yellowish	Antenna with 6 funicular segments, the last atrophied, clava is bisegmented

Sampling sites and wasp collection

Two sampling sites were selected in Xishuangbanna in tropical SW China. One site was located in the public square of Jinghong city where five trees of *F. altissima* are growning. The other site was located in Menglun town, about 70 km East of Jinghong city. In this area, two *F. altissima* trees were located in Xishuangbanna Tropical Botanical Garden and eight were found ~3 km away in Chengzi village.

The 15 studied trees were observed every two weeks from January 2003 to December 2005. When available, 30 figs at the wasp emerging phase were collected from each tree. Figs were placed individually in fine-mesh bags (200×200 mm), and the fig wasps allowed to emerge. All fig wasps, including wingless males, were carefully collected and preserved in 70% ethanol. Each wasp was identified to species and counted. During the three years of the survey, we sampled 46 fig crops (fig samples from a single fruiting event from a given tree). Of the sampled figs (n=1380), 21 did not contain any wasps. The remaining 1361 figs contained at least one of the 25 fig wasp species, while 414 produced only *Eupristina* species. As the number of *Eupristina* may be affected by the presence of non-pollinating fig wasps (Kerdelhué and Rasplus, 1996; Kerdelhué et al., 2000; West et al., 1996), we used only the 414 figs entered by *Eupristina* alone to calculate the relative abundance of each species and a sub-sample of 375 figs were used to compare the number of offspring emerging. In 2004 and 2005, we also recorded the number of seeds produced per fig. Consequently, we had 209 figs to compare the pollination efficiency of each *Eupristina* species. Because we were unable to separate males of the two *Eupristina* species, we assummed, when we found females of only one species in a fig, that the males belonged to the same species.

Experimental introductions of Eupristina *spp.*

From March to May 2004, a suitable tree was located, and 40 pre-receptive figs were selected for experimental introduction of *Eupristina* spp. The twig bearing each fig was encased in a fine-mesh nylon bag (200×200 mm). Each bag was sealed tightly around the twig to prevent any fig wasps arriving naturally at the tree to pollinate the fig. Simultaneously, mature figs were collected on trees in the vicinity, stored in nylon bags and the fig wasps were allowed to emerge. Twenty females from each species were identified under a binocular microscope and we introduced one female to each of the 40 sealed bags containing one receptive fig. The bags were left on the figs. When the figs were mature, we collected the fig wasps, according to the method explained above. For each fig, the number of *Eupristina* and seeds produced were recorded.

Ventral views of female Eupristina *mesosoma*

In order to detect whether both *Eupristina* species pollinated actively, we took SEM photos of the pollen pockets and corbicula. Pollen pocket and corbicula on ventral mesosoma are two traits associated with the mode of pollination in agaonid fig wasps. Coxal comb is considered as the most reliable trait for inferring mode of pollination, as many species of agaonids still possess pollen pockets but do not actively collect and deposit pollen (Kjellberg et al., 2001). However, in such cases the pockets are usually reduced and so the character is useful for assessing the ancestral state in non-pollinating (e.g. *Ceratosolen galili*) or passively pollinating agaonids.

3. Results

Do both Eupristina *species occur on the same tree at the same time?*

In Xishuangbanna, *F. altissima* produced figs all year-round, with more trees bearing figs in March and June. The trees under observation flowered from 0 to 6 times during the three year period of observation. Fewer trees flowered in 2005, possibly as a result of a severe drought, only four crops at JingHong and three crops at Menglun were produced. Among the 46 crops we sampled, we found both *Eupristina* species in 36 (79%), only *Eupristina* sp. in 8 (17%), and only *E. altissima* in 2 (4%) (Fig. 1).

Of the 414 figs sampled that had only *Eupristina* spp., 62% contained only *E. altissima*, 29% contained only *Eupristina* sp., and 9% contained both species (Fig. 2).

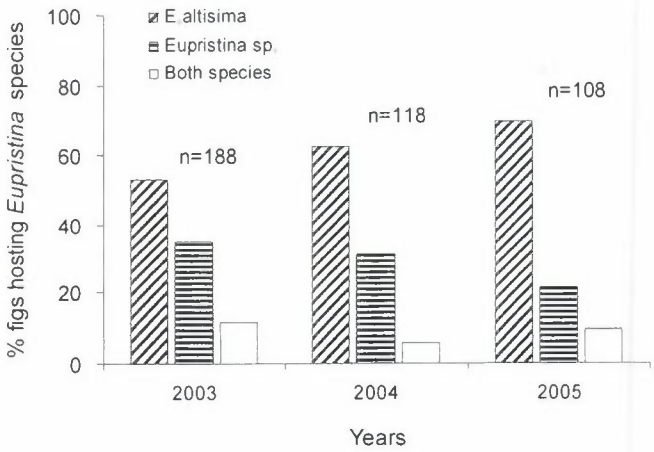

Figure 2. Percentage of *F. altissima* figs containing *Eupristina altissima*, *Eupristina* sp., or both species over a three year period in Xishuangbanna, SW China.

Can both Eupristina *species successfully reproduce in the figs of* F. altissima*?*

In the natural community, 57% of sampled figs contained one foundress, and an average of 1.8 foundresses entered a fig to oviposit. At eclosion, the average numbers of *Eupristina altissima* emerging from a fig was significantly lower than that of *Eupristina* sp., although the amplitude of the difference was small (*E. altissima* 203.05±58.18, and *Eupristina* sp. 220.53±93.70; Mann-Whitney *U* Test P=0.04) (Fig. 3). When one foundress was introduced and entered a fig to oviposit,

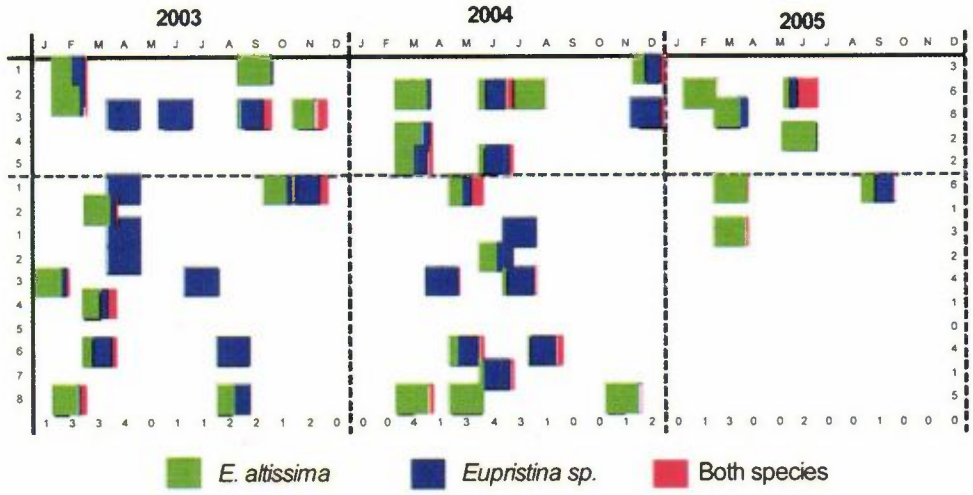

Figure 1. Co-occurrence of two *Eupristina* species on *Ficus altissima* in Xishuangbanna, SW China. Numbers on the left identify the trees. The five upper trees are in the public square of Jinghong city, and two middle trees are located in Xishuangbanna Tropical Botanical Garden, and eight bottom trees are found in Chengzi village. Numbers on the right give the total number of times a tree flowered during the three years survey, and numbers along the bottom give the total number of trees flowering in each month. The different colors represent colonization of figs by each *Eupristina* species separately and together, respectively. The area of shading is in proportion to the abundance of the figs in each case.

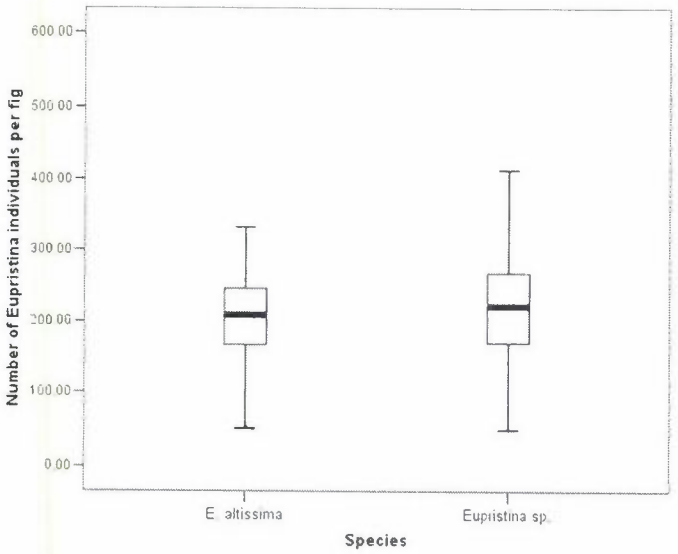

Figure 3. Numbers of individuals of two species of *Eupristina* per fig emerging from *F. altissima* in Xishuangbanna, SW China. There was a significant difference between the mean number of wasps produced (Mann-Whitney Test, P=0.04).

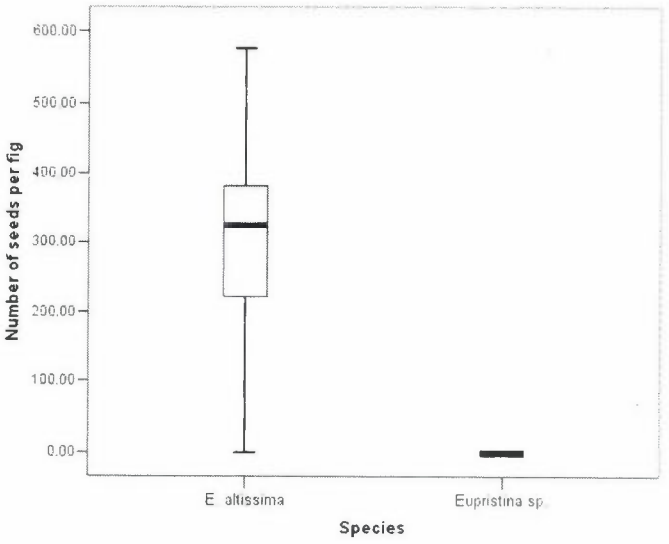

Figure 5. Seed production in *F. altissima* figs colonized by either *Eupristina altissima* or *Eupristina.* sp. in a natural population in Xishuangbanna, SW China. There was a highly significant difference between the mean number of seeds produced (Mann-Whitney Test, P<0.001).

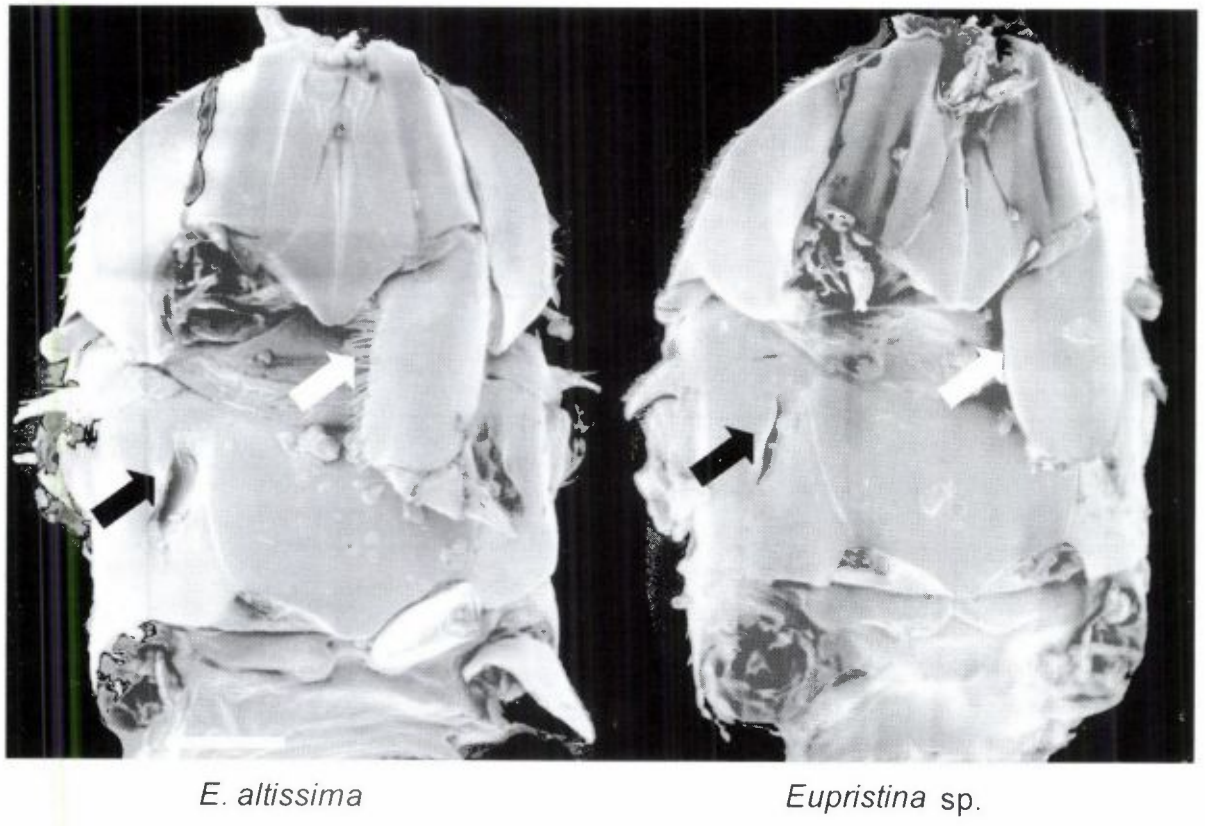

Figure 4. Ventral views of female (left) *Eupristina altissima* and (right) *Eupristina* sp. mesosoma. Black arrows indicate the pollen pockets and white arrows indicate the coxal comb.

Eupristina altissima produced lower offspring (197.22±52.27) than *Eupristina* sp. (201.25±78.21), but there was no significant difference between the numbers of offspring produced by the two *Eupristina* species (Mann-Whitney *U* Test, P=0.76).

Are both species of Eupristina *effective pollinators of* F. altissima?

The mode of pollination occuring in a species of *Ficus* can be consistently predicted from the anther-to-ovule ratio (Kjellberg et al., 2001). On a representative number of fig species, these authors showed that an anther-to-ovule ratio less than 0.16 indicates active pollination, while a ratio over 0.20 is characteristic of passively pollinated species. We counted the numbers of anthers and female flowers in figs of *F. altissima* (n=60) and showed that the average anther-to-ovule ratio was 0.09; a value characteristic of active pollination. Observation of wasp morphological traits correlated with pollination mode (presence or absence of pollen pockets and coxal comb) indicating that *E. altissima* is an active pollinator. It has well developed pollen pockets and coxal comb (Fig. 4). In contrast, *Eupristina* sp. did not exhibit a comb of setae on the fore coax, and the pollen pockets were almost closed (Fig. 4). These characters strongly suggest that *Eupristina* sp. is not an active pollinator.

To examine the pollination efficiency of *E. altissima* and *Eupristina* sp. in the figs of *F. altissima*, we counted the number of seeds produced in figs apparently colonized by only one of these species. Altogether, we collected 209 figs from which only one *Eupristina* species emerged (*E. altissima* = 149, *Eupristina* sp. = 60). Figs colonized by *E. altissima* produced on average 295.38±118.37 seeds, while figs colonized by *Eupristina* sp. produced on average 14.35±50.65 seeds (Fig. 5), a highly significant difference (Mann-Whitney *U* Test, P<0.001). Within the 60 figs colonized only by *Eupristina* sp., 82% had no seed production. These results strongly corroborated the prediction, based on the wasp morphology, that *Eupristina* sp. is not an active pollinator and is an ineffective pollinator of *F. altissima*. From our 40 controlled introductions (20 for each species), we were only able to obtain 13 successful introductions, 9 figs with *E. altissima* and 4 figs with *Eupristina* sp. The figs entered by *E. altissima* had 221.22±54.76 seeds, while those entered by *Eupristina* sp. had only 2.50±5.00 seeds (Mann-Whitney *U* Test, P=0.005). Among the four figs entered by *Eupristina* sp., three were not pollinated at all, and there were only 10 seeds inside the last fig.

4. Discussion

The fig-fig pollinator mutualism was formerly considered strictly species-specific, with every fig species pollinated by a single fig wasp species, and each wasp species reproducing in only one fig species (Ramirez, 1970; Wiebes, 1979; Berg and Wiebes, 1990). However, recent studies clearly show that many fig species host at least two pollinating fig wasps (Kerdelhué et al., 1997; Lopez-Vaamonde et al., 2002; Molbo et al., 2003; Machado et al., 2005).

Ficus altissima hosts two species of *Eupristina* in Xishuangbanna, SW China. This study shows that these species differ in their ability to pollinate *F. altissima*. *Eupristina altissima* is an effective pollinator of *F. altissima*, but *Eupristina* sp is a "cuckoo" of the mutualism. This situation is similar to that observed in a cuckoo agaonid species, *Ceratosolen galili*, in *F. sycomorus*, where low number of seeds were produced in figs colonized only by *C. galili* (Compton et al., 1991). In *Ficus altissima*, most of the figs (>80%) from which only *Eupristina* sp. emerged produced no seeds, and the others produce very few seeds. *Eupristina sp.* does not have a coxal comb and has small, probably non functional, pollen pockets. Therefore, either accidental pollination sometimes occurs or perhaps these figs were in fact pollinated by an *E. altissima* individual that did not produce offspring, or produced only male offspring (i.e. she was an unfertilized female). Another possibility, given the very small numbers of seeds produced in these figs, is that the seeds resulted from pollen passively carried on the body of the *Eupristina* sp. foundresses. An important piece of evidence that would enhance our knowledge of the biology of *Eupristina* sp would be the direct observation of ovipositing behavior. It is conceivable that *Eupristina* sp. could be an active, but ineffective pollinator of *F. altissima*. However, the absence of coxal combs and reduced pollen pockets make this a remote possibility.

It is interesting that 17% of *F. altissima* crops and 29% of the figs were inhabited only by *Eupristina* sp. It indicates that *Eupristina* sp. does not require pollination to complete its larval development.

When a fig was colonized by only one species, the average number of *Eupristina* sp. emerging from figs was higher than that of *E. altissima*. This result leads to several conclusions:

- Seed production is not obligatory to sustain wasp development. This result agrees with previous findings obtained from other species of *Conosycea* (Jousselin et al., 2003; Harrison, 2007).
- The development of *Eupristina* sp. alone, and consequently in the absence of pollination, did not lead to fig abortion, as again reported from other *Conosycea* species (Harrison, 2007).
- In some situations, there is no fitness cost if an active pollinator fails to pollinate. These conditions can permit a shift from mutualism to parasitism (Sachs and Simms, 2006).

In the sole other case, documented to date, of reversal to parasitism in Agaonidae (*C. galili* on *F. sycomorus*), the two *Ceratosolen* species are not sister species (Wiebes, 1989; Kerdelhué et al., 1999). However, morphological evidence strongly suggests that *E. altissima* and *Eupristina* sp. belong to a species complex and are closely related. They may even be sister taxa. This needs to be demonstrated by using molecular data and through systematic collections on related *Ficus* species.

It appears cheaters are not common in the fig-fig pollinator mutualism, but the discovery of more and more cases of multiple pollinator species co-existing on a single host species makes it likely to be more frequent than currently reported. Active pollination seems to have evolved from ovule parasitism. Our study demonstrates a second case of reversal to ovule parasitism from active pollination. However, our understanding of the ecological and evolutionary conditions that lead to the stable coexistence of mutualistic and cheating agaonids in *Ficus altissima* remains limited and needs further investigation.

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