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Reproductive success and pollination of the Tuncurry Midge Orchid (*Genoplesium littorale)* (Orchidaceae) by Chloropid Flies

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Abstract

The Midge Orchids (*Genoplesium* R.Br.) (Orchidaceae) are thought to attract pollinators by nectar reward. All verified records of *Genoplesium* pollinators are small flies of the families Chloropidae and Milichiidae, suggesting pollinator specificity. We investigated pollination of the Critically Endangered Tuncurry Midge Orchid, *Genoplesium littorale* D.L.Jones. In common with other *Genoplesium* species, *G. littorale* is pollinated exclusively by chloropid flies. Although there is specificity at the pollinator family level, *G. littorale* is oligophilous, being pollinated by five putative chloropid species in two genera, *Conioscinella* and *Cadrema*. Most visitors were female with females greatly predominating among flies bearing pollinaria. Examination of flowers on ten inflorescences showed *G. littorale* is outcrossing with high pollen vector activity; pollinaria had been removed from 71% of post anthesis flowers. A set of criteria for distinguishing outcrossed, autogamous and apomictic flowers based on observations of pollinaria removal, pollination of stigmas and fruit set on individual flowers ruled out the occurrence of autogamy and apomixis in *G. littorale*. Fruit set on inflorescences averaged 44% prior to seed dispersal and varied significantly among sub-populations. Nectar is produced in the groove of the labellum callus, although flowers emitted no odour detectable by humans. Detailed examination of 29 flowers revealed no chloropid eggs, indicating the pollination syndrome is not brood site mimicry. The absence of strong dung or carrion-like odours also makes sapromyophily unlikely. The *Genoplesium* pollination syndrome is nectar reward, but may also represent an example of 'kleptomyiophily', recently described in *Aristolochia rotunda*. Herbivory reduced reproductive capacity by half overall, varied significantly among sub-populations and may be a significant threatening process for *G. littorale*. Strategies to reduce herbivory in this critically endangered species should be investigated.

Introduction

Orchids are renowned for their floral diversity, reflecting a variety and complexity of pollination mechanisms that have fascinated naturalists since Darwin's pioneering investigations (Darwin 1877, Edens-Meier and Bernhardt 2014). The majority of orchids are thought to be pollinated via nectar reward of insect pollen vectors (van der Pijl and Dodson 1966). However, orchids are also known for the relatively high proportion of species with deceptive pollination strategies. Up to one third of orchid species deceive pollen vectors by promising non-existent rewards (van der Pijl and Dodson 1966). It is well known that orchid pollen vectors may respond to false offers of nectar (food deception) or a mate (sexual deception) (Vereecken *et al.* 2010).

More recently, some orchids have been shown to mimic carrion (van der Niet *et al.* 2011), or various kinds of prey insects including aphids (Stökl *et al.* 2011), leaf-eating caterpillars (Brodmann *et al.* 2008) and honey bees (Brodmann *et al.* 2009).

Despite an absence of formal scientific studies, the dominant pollination mechanism in the Australian terrestrial orchid genus *Genoplesium* Fitzg., commonly known as Midge Orchids, is considered to be xenogamy or geitonogamy mediated by small Diptera (Bower 2001a). Most of the 55 described *Genoplesium* species are thought to reward fly pollinators with nectar. However, three species, *G. nudum* (Hook.f.) D.L.Jones & M.A.Clem., *G. pumilum* (Hook.f.) D.L.Jones & M.A.Clem. and south-west Tasmanian *G. archeri* (Hook.f.) D.L.Jones & M.A.Clem., are considered to be autogamous (Jones 1972, 1998), and *G. apostasioides* Fitzg. is apomictic (Jones 1977, Jones and Clements 1989). Entomophilous *Genoplesium* species may strongly attract swarms of flies on warm days (Bates 1981, 1988). The primary attractant appears to be odour, with some scents detectable by humans, but others not so. Reported *Genoplesium* odours include lemon, lemon mixed with an ant-like odour and sour milk (Blaxell 1970, Jones 1970). Garnet (1940) reported that the labellum (the specialized median petal of orchids) callus of newly opened *Genoplesium* flowers is liberally covered with 'minute droplets of glandular exudate'. No other reports mention the presence of nectar-like secretions in Midge Orchid flowers.

The mechanism of insect mediated pollination in *Genoplesium* was first described by Garnet (1940). Flies land on the inflorescence and move to the downward hanging labellum which they gradually ascend, whilst appearing to feed. Once on the labellum flies are oblivious to close observation with a hand lens or even inversion of the inflorescence (Garnet 1940, Bates 1981). They move to the labellum base (Garnet 1940) and may force the thorax below the rostellum by jerking movements of the legs (Bates 1981, 1988) where they spend up to several minutes. In this position the fly's thorax contacts the viscidium. After finishing on one flower flies may move to others on the same raceme (Garnet 1940) suggesting geitonogamous self-pollination occurs. Because they remain on the inflorescence rather than departing, this behaviour also suggests flies derive a reward (Bates 1988).

Despite reports that Vinegar Flies (Drosophilidae) may pollinate *Genoplesium* species (Jones 2006, Kuiter 2013), all verified records of visitors to *Genoplesium* species involve flies of the families Chloropidae and the closely related Milichiidae (Bower 2001a). The following collates all known records of chloropid visitors to *Genoplesium* species with nomenclature updated in accordance with Evenhuis (2012). Chloropid specimens collected by Garnet (1940) belonged to up to five species in three genera and two families, but only *Caviceps flavipes*, *Gaurax subpilosa* (as *Oscinosoma subpilosa*) and an undescribed species of *Gaurax* (as *Oscinosoma*) were named. The orchid species visited by each fly species were not given. Cady and Rotherham (1970) illustrated the chloropid *Conioscinella beckeri* carrying pollinia on the labellum of *Genoplesium archeri*. Chloropid flies collected by D. L. Jones (unpublished) on various *Genoplesium* species were identified as species of *Caviceps* on *G. nigricans* (R.Br.) D.L.Jones & M.A.Clem., *G. despectans* (R.Br.) D.L.Jones & M.A.Clem., *G. morrisii* (Nicholls) D.L.Jones & M.A.Clem. and *G. rufum* (R.Br.) D.L.Jones & M.A.Clem.; *Caviceps flavipes* was also collected on *G. rufum* (Bower, 2001a). Flies captured by A. E. Logan (unpublished) on *G.* aff. *rufum* were identified as *Gaurax* sp. (as *Lioscinella* sp.) (Chloropidae) and *Stomosis* sp. (Milichiidae) (Bower 2001a). The limited data suggest that entomophilous *Genoplesium* species may be exclusively myophilous and specifically adapted to chloropids and milichiids for pollination.

It is not clear if there is pollinator specificity between individual *Genoplesium* species and chloropid species. Garnet (1940) did not report which species of flies were attracted to each *Genoplesium* species, but considered pollinators were shared among species. By contrast, observations by Jones (1970) and Bates (1988) suggest some level of specificity may occur. Jones (1970) observed that attracted flies behaved differently towards five species of potted *Genoplesium* placed together in a backyard. The flies removed the pollinaria of only one species, *G. morrisii*, but also actively worked the flowers of *G. despectans*. They landed on the inflorescence of *G. fimbriatum* (R.Br.) D.L.Jones & M.A.Clem., but did not enter the flowers, and showed little interest in *G. nigricans* (as *Prasophyllum fusco-viride*). The flies ignored *G. filiforme* (Fitzg.) D.L.Jones & M.A.Clem. (as *P. nublingii* R.S.Rogers) altogether. Similarly, Bates (1981) observed that flies visiting *G. ciliatum* (Ewart & B.Rees) D.L.Jones & M.A.Clem. were larger than those visiting *G. nigricans* and *G.* aff. *rufum* in the same glasshouse over the same time period. However, Bates (1988) also found that the same unidentified fly species visited *G. acuminatum* (R.S.Rogers) D.L.Jones & M.A.Clem. and *G. ciliatum* in the same glasshouse.

While the roles of flies (Diptera) as pollinators of angiosperms has been long recognised, being considered the second most import order of insects for pollination after the Hymenoptera (Larson *et al.* 2001), small flies, including both the Chloropidae and Milichiidae, are rarely recorded visiting flowers and their association with flowers is often overlooked (Larson *et al.* 2001). The Chloropidae and Milichiidae have been identified as pollinators of species from several families in addition to the Orchidaceae including the Apocynaceae

(including Asclepiadaceae), Aristolochiaceae and Araceae (Proctor *et al*, 1996, Borba and Semir 2001, Heiduk *et al.* 2010 and Oelschlägel *et al*. 2014). The known mechanisms by which myophilous flowers within these families attract flies is discussed below.

Most myophilous flowers reward attracted flies with food, either nectar or pollen (Faegri and van der Pijl 1966, Proctor and Yeo 1973). However, several plant families, notably the Aristolochiaceae, Apocynaceae, Araceae and Orchidaceae employ various deceptive mechanisms to lure flies for pollination (Proctor *et al.* 1996). The most prominent mechanism is brood site imitation in which pollinators are stimulated to lay eggs on tissues that mimic fungi or carrion, but on which the pollinator's offspring cannot survive (Proctor *et al*, 1996). Many deceptive fly pollinated flowers in the Aristolochiaceae and Araceae are protogynous with elaborate trap mechanisms that detain flies, sometimes for up to 24 hours, while the flower transitions between female and male phases, first being pollinated and then releasing pollen onto the same flies after the stigmas are no longer receptive (Proctor *et al*, 1996). Heiduk *et al*. (2010) suggest that the floral scent of C*eropegia dolichophylla* (Apocynaceae), which was found to include known insect alarm pheromones, attracts kleptoparasitic milichiid flies as pollinators by mimicking their feeding sites on dead and dying insects. Recently, Oelschlägel *et al*. (2014) demonstrated pollination of the trap flower *Aristolochia rotunda* mainly by deception of female kleptoparasitic chloropids that normally feed on the leaking hemolymph of mirid bugs freshly killed by predatory insects or spiders. The chloropids are attracted by volatiles emitted by the dying bugs; the same chemicals are also emitted by *A. rotunda* in a pollination syndrome they termed 'kleptomyiophily' (Oelschlägel *et al*. 2014). Chloropids of the genera *Tricimba* and *Hippelates* are the exclusive pollinators of two lithophytic *Pleurothallis* orchid species (*P. johannensis* and *P. fabiobarrosii*) in eastern Brazil (Borba and Semir 2001). Only females of *Tricimba* sp. removed and deposited pollinaria, and were frequently observed to oviposit on flowers, although fly larvae and pupae were not seen. The two *Pleurothallis* species lack nectar, imitate *Tricimba* brood sites and are pollinated by brood site deception (Borba and Semir 2001).

The subject of this study, the Tuncurry Midge Orchid, *Genoplesium littorale*, is listed as Critically Endangered (NSW Scientific Committee 2009, Threatened Species Scientific Committee 2011). It is known only from the Forster-Tuncurry area on the New South Wales North Coast. The total population is estimated at approximately 2000 plants distributed across an area of 8 km^2, mainly on consolidated sand dunes and rehabilitated sand mining paths (Threatened Species Scientific Committee 2011). Its reproductive strategy, pollination success and pollinators, if any, are unknown. The study reported here was undertaken to determine the pollination mechanism, pollinators and reproductive success of *G. littorale*, in order to better conserve it through an increased understanding of its ecological requirements (Dixon *et al.* 2003, Swarts and Dixon 2009).

The literature suggests *G. littorale* is likely to be outcrossing and pollinated by chloropid or milichiid flies. However, some species of *Genoplesium* are autogamous or apomictic (Jones 1972, 1988) and there are a number of other myophilous pollination strategies that may apply to *G. littorale*. While most myophilous plants attract pollinators by nectar or pollen rewards, various deceptive mechanisms are known including; brood site mimicry (Borba and Semir 2001), mimicry of decaying organic matter (sapromyophily) (van der Niet *et al.* 2011) or, rarely, mimicry of the dying prey of predatory insects and spiders (Oelschlägel *et al*. 2014).

Accordingly, the study aimed to determine whether *G. littorale*:

is autogamous, apomictic or entomophilous;

is pollinated by chloropid and/or milichiid flies;

is monophilous, oligophilous or polyphilous, if entomophilous;

is nectar rewarding;

has brood sites for pollinators; or

has characteristics of sapromyophily or kleptomyiophily.

The study also aimed to estimate the pollination and reproductive success of *G. littorale*.

Methods

Study Species: *Genoplesium littorale* is a renascent terrestrial herb, with a single tubular leaf to 25 cm high from which emerges the single flower stem bearing from 5 to 30 small (5 × 4 mm) yellowish green flowers with dark reddish black extremities. A distinctive feature is the fleshy, purplish brown labellum with a prominent furrowed callus. All floral segments lack marginal hairs. Flowering occurs between March and May, after which plants die back to the underground tuber. A new leaf emerges with soaking rains in late summer.

Genoplesium flowers are resupinate and possess a pollinarium in which four sectile (friable) pollinia in two pairs are joined to a viscidium via a common stipe (Jones 2001). The pollinarium ensures that all pollen is removed from the anthers by a single successful visit by a pollen vector (van der Pijl and Dodson 1966). No bending of the stipe occurs after pollinarium removal because the anthers are located behind the stigma and the pollinia are consequently correctly oriented upon removal for contacting the stigma of another flower. The sectile pollinia may pollinate multiple flowers by leaving fragments of pollen on the stigmas of successive flowers visited. In common with most orchids (O'Neill 1997, van Doorn 1997), the flowers of *Genoplesium* close rapidly and wither within one or two days of being pollinated, followed by rapid swelling of the ovary to form the fruit capsule.

Study area, timing and plant labelling: The study was undertaken at the main known population sites of *G. littorale* north of Tuncurry (precise location details are not given due to the species rarity). Preliminary observations were made at the end of the flowering season in 2012 with the main study taking place in 2013. Additional pollinator collections were made in 2014.

In 2013, 141 plants were individually labelled with small plastic horticultural tags placed in the soil 10 cm from each plant with the label facing the plant. The presence of closed (post anthesis) flowers, open flowers and buds was recorded at labelling. Plants were tagged in four sub-population groups, mainly on March 12 and 13 (Table 1). Labelled plants were monitored for pollinator visitation and used to measure overall reproductive success prior to seed dispersal. The same groups of plants were used for additional pollinator collections in 2014.

Table 1. Groups of *Genoplesium littorale* study plants for pollinator observations (12–14 March 2013 and 18–21 March 2014) and fruit set assessment (23 April 2013), indicating generalised locality and number of plants studied.

Group	Location	No. of plants
A	Chapmans Road	34
B	South of North Boundary Fire Trail	20
C	North of North Boundary Fire Trail	57
D	South of track to Darawank Nature Reserve	30
Total		141

Pollinator observations and capture: Flowers of individual labelled plants in each group were examined closely for the presence of insects and whether they were carrying the distinctive *Genoplesium* pollinarium. The only other co-flowering species, *Chiloglottis diphylla* Fitgz., was restricted to Group D and does not form a pollinarium; its pollinia lack stipes and a viscidium. Each plant was observed for approximately 10 to 15 seconds three or four times daily on 12 to 14 March 2013 and 18 to 21 March 2014.

Insects were captured with a manual aspirator and preserved in 70% ethanol (during study of 2013), or frozen (2014). Captured insects were identified by one of us, D. Bickel. Owing to the lack of comprehensive taxonomic treatments of the Australian Chloropidae, specimens were segregated into informal species based on robust taxonomic characters used widely in the family. Genus level identification was based on the most recent international key (Wheeler 2010). All insect specimens have been lodged with the Australian Museum, Sydney.

Determination of pollination strategy: Pollinator exclusion by caging inflorescences, and observing whether fruit and viable seed develop, is commonly used to demonstrate the existence of autogamy or apomixis (with emasculation) (Dafni *et al.* 2005). Caging experiments are time-consuming and costly. In addition emasculation is not practical in *G. littorale* owing to its numerous small flowers that do not open synchronously. Caging was considered unnecessary for *G. littorale* following microscope examination of three inflorescences collected late in the 2012 flowering season. These observations suggested *G. littorale* is obligately outcrossing and that autogamy and apomixis do not occur. Vector-mediated pollination, autogamy and apomixis can be distinguished morphologically in flowers developing fruit by different combinations of pollinaria removal, presence of pollen on the stigma and fruit development (Table 2). In the case of autogamy physical evidence of pollen or gamete transfer from anther to stigma within the flower is also necessary, since it is possible for outcrossing flowers to be pollinated by a visiting insect without pollinaria removal occurring.

Table 2. General pollinarium, stigma and fruit set status of flowers exhibiting outcrossing, autogamy and apomixis (some outcrossed flowers may share the combination for autogamy, in which case morphological evidence for autogamy is also needed).

	Outcrossing	Autogamy	Apomixis
Pollinarium removed	✓	X	X
Stigma pollinated	✓	✓	X
Most viable flowers develop fruit	X	✓	✓

Morphological evidence for autogamy sought through examination of flowers included the following. The pollinarium must be *in situ*, to allow growth of pollen tubes into the stigma from the anthers, or the spilling of pollen from disintegrating pollinia onto the stigma after the column has bent forward (Jones, 1972), or by outgrowth of the stigma to contact the pollinia (Jones, 1972). Evidence necessary to demonstrate apomixis is the presence of swollen seed capsules in combination with a lack of pollen on all stigmas and the pollinia remaining *in situ* in the anther sacs without breaking down or germinating. Both autogamy and apomixis result in the development of fruit capsules in a high proportion of flowers (Neiland and Wilcock 1998).

Ten inflorescences of *G. littorale* with closed flowers, open flowers and buds, three in 2012 and seven in 2013, were examined using a binocular dissecting microscope at magnifications up to 40 times. The presence or absence of the pollinarium in the anthers and of pollen on the stigma was recorded for each flower, along with its status (open, closed or unopened bud). Absence of the pollinarium in open or closed flowers is evidence of removal by a pollen vector. The percentage of empty anthers on each inflorescence provides a measure of visitation by pollinators and the percentage of stigmas carrying pollen grains measures pollination levels, in the absence of autogamy.

Pollination success: In this study, as in many ecological studies on the Orchidaceae, pollination success was measured post anthesis by the percentage of flowers setting fruit on each inflorescence [developing infrutescence] (Neiland and Wilcock 1998, Tremblay *et al*. 2005). In *Genoplesium*, and most other orchids, there is a marked difference between the distension of fertilised versus unfertilised ovaries (Fig. 1). Accordingly, it was not considered necessary to assess seed viability as a measure of reproductive success. Fruit set was assessed on 23 April 2013 on 131 remaining labelled plants. Of the original 141 labelled plants, seven inflorescences were removed for laboratory examination and three labels were lost.

Presence of nectar and brood sites: The presence or absence of nectar droplets in the labellum groove was recorded from 10 harvested inflorescences, three in 2012 and seven in 2013 (the same 10 inflorescences taken for determination of pollination strategy). Inflorescences were examined with a 10× hand lens after picking for the presence of nectar on the labellum. The labellums of one flower on six of the inflorescences were photographed with a macro lens and later digitally magnified on a computer screen as a further check for the presence of nectar. Inflorescences with open flowers were smelt in warm conditions to determine if 'food' odours similar to those reported by Blaxell (1970) and Jones (1970) for other *Genoplesium* species were present, or whether odours associated with sapromyophily, such as dung or carrion-like smells were present. The possible presence of brood sites was tested by microscopic examination of single flowers taken from 29 plants; Group A (7 flowers), Group B (9 flowers) and Group C (13 flowers) in 2014. Flowers were stored in individual vials at 3°C and examined within two days of collection. All tepals and the column were inspected thoroughly.

Statistical analysis: Statistical analyses were conducted in *WinSTAT for Excel* (Fitch 2002).

Results

At the time of plant labelling in 2013, 73% of plants had open flowers and 27% were in bud with no open flowers. Fruit capsule development had commenced with swollen ovaries present on 9 percent of plants. Thirty percent of plants had closed (post anthesis) flowers without swollen ovaries. Unopened buds were present on 51% of plants with open flowers.

Pollinators: Maximum temperatures on the study area were 28.9, 28.1 and 30.0 °C on 12, 13 and 14 March 2013, respectively, which are at the upper end of the optimal range for insect activity (Taylor 1963). Fifty one potential pollinators, all small flies, were observed on *G. littorale* inflorescences in 2013. Nineteen (38%) were carrying pollinaria on the dorsal thorax. Twenty two were captured and identified, including ten bearing pollinaria. A further 33 flies were captured in 2014, of which 16 (48.5%) were carrying pollinaria (Fig. 2). The flies belonged to five morphotypes in the family Chloropidae that are considered to be putative species here.

They keyed to two genera, *Cadrema* (1 taxon) and *Conioscinella* (4 taxa) (Table 3). The Australian Chloropidae is a neglected group with many undescribed species, and it was not possible to identify the specimens beyond the generic level. The characters used to distinguish the five informal taxa are consistent among the material examined (Table 3).

Specimens of all five putative chloropid species carried *G. littorale* pollinaria on the thorax (Table 4, Fig. 2), thereby confirming them as *G. littorale* pollinators. Twenty specimens carried a single pollinarium, and six bore two pollinaria. Most of the flies captured were females; 39 females to 16 males, which deviated significantly from the expected 1:1 ratio (P=0.002, chi square test). The disparity between females and males was greater among flies with pollinaria; 23 females to 3 males (P=0.0001, chi square test).

Table 3. Distinguishing characters of five putative species of chloropids attracted to *Genoplesium littorale* at Tuncurry, New South Wales.

Chloropid taxon	No. of specimens	Distinguishing characters
Cadrema sp. 1	2	Tibia III with long curved apical spine; subrectangular antenna
Conioscinella sp. 1	19	Distal frons and gena yellow; tibia II & III with banded appearance; antenna yellowish
Conioscinella sp. 2	5	Distal frons yellow; antenna dark brown
Conioscinella sp. 3	8	Distal frons black; antenna rounded, yellow; very small, < 1.0 mm
Conioscinella sp. 4	21	Distal frons black; antenna black

The two most common chloropids in the collection, *Conioscinella* sp. 1 and *Conioscinella* sp. 4, were also the species with most pollinaria (Table 3), suggesting they are the dominant pollinators of *G. littorale* on the study area. While *Conioscinella* sp. 2 and *Conioscinella* sp. 3 were less common, they nevertheless contributed to pollination of *G. littorale* (Table 3). Although uncommon, both *Cadrema* sp. 1 specimens bore pollinaria, indicating this species is an effective pollinator.

Table 4. Chloropid visitors to *Genoplesium littorale*: taxa, sex, presence of pollinaria and area of capture.

Chloropid taxon	12-14 March 2013			18-21 March 2014	
	Sex	No. with pollinaria	Area	Sex	No. with pollinaria
Cadrema sp. 1	2♀	2	A, C	-	-
	0♂	-	-	-	-
Conioscinella sp. 1	8♀	5	A, B, C, D	3♀	1
	3♂	0	B, D	5♂	1
Conioscinella sp. 2	1♀	0	B	1♀	1
	1♂	0	B	2♂	0
Conioscinella sp. 3	5♀	2	B, C, D	2♀	1
	1♂	1	D	0♂	-
Conioscinella sp. 4	1♀	0	A	16♀	11
	0♂	-	-	4♂	1
Totals	17♀	9		22♀	14
	5♂	1		11♂	2

Pollinarium position: Attachment of the *G. littorale* viscidium to chloropids is nototribic (attached to the mid-thoracic region) (Fig. 2). The viscidium contacts the mid line of the thorax as the fly straddles the labellum groove seeking nectar. As the fly moves towards the labellum base it contacts the viscidium which adheres to the thorax (Fig. 2). The precise position of the viscidium depends on the size of the fly and whether it has already picked up a pollinarium from another flower. In flies with single pollinaria, the viscidium was centred on the bilateral centre line of the dorsal thorax, but may occasionally be placed just to the side of the centre line. Flies with two pollinaria had one straddling the centre line and the other displaced to the right or left side; either immediately to the side, or forward and to the side. The position of the pollinarium varies anteroposteriorly on the midline from the centre of the mesonotum to the rear of the mesonotum, in some cases extending partially onto the scutellum.

Fig. 1. Post anthesis inflorescence of *Genoplesium littorale* showing swollen seed capsules (fruit) and unfertilised flowers.

Fig. 2. *Conioscinella* sp. 1 (left) and *C.* sp. 4 (right) showing nototribic pollinarium position.

Weather conditions: Pollinators were observed and captured between 8:30 and 16:20 hours on 12 to 14 March, 2013. Temperatures varied between 19.8 and 30.0 °C with most flies (78.7%) observed when temperatures were above 25 °C (Fig. 3) and after midday (74.5%). Pollinators were active in both sunny and light to medium cloudy conditions.

Odour and nectar: No food-like, or dung or carrion-like odours were detected from *G. littorale* inflorescences in warm conditions (25 to 30 degrees C) when pollinators were most commonly encountered on the flowers. A line of nectar droplets in the groove of the labellum callus plate (Fig. 4) was revealed when the labellum of one flower macro-photographed in the field was digitally magnified on a computer screen, but nectar was absent on five others. In addition, liquid, including lines of droplets similar to Fig. 4, was observed on the labella of half the 29 fresh flowers (55.2%) that were refrigerated for one to two days prior to microscope examination for possible fly egg deposition. Moisture was not found on other floral segments and is unlikely to have been condensation.

Flower examinations: Ten harvested inflorescences carried a total of 141 flowers of which eight were buds, 50 were open and 83 had closed (Table 5). Examination of flowers showed that in all cases whole pollinaria were taken from the anthers. High levels of pollinaria removal had occurred in some plants, up to 90 percent (varying from no pollinaria removed to 90% removed). The percentage of pollinaria removal in 83 post anthesis flowers was 71.1%. Pollen grains were found on the stigmatic surface of 98% of flowers that had developed fruit.

The pollinaria had been removed from half the open flowers and 12% had pollen on their stigmas (Table 5). Orchid flowers generally close within one to two days of pollination (O'Neill 1997, van Doorn 1997), explaining the low number of open flowers with pollen on their stigmas. Following closure the ovary begins to swell as fertilised seeds develop. Among the 29 post anthesis flowers with unswollen ovaries, three with pollen on their stigmas had not yet commenced swelling (Table 5). Fifteen other unswollen post anthesis flowers had intact pollinaria retained in the anthers and no pollen on their stigmas. The failure of any of these flowers to develop fruit indicates autogamy and apomixis are absent.

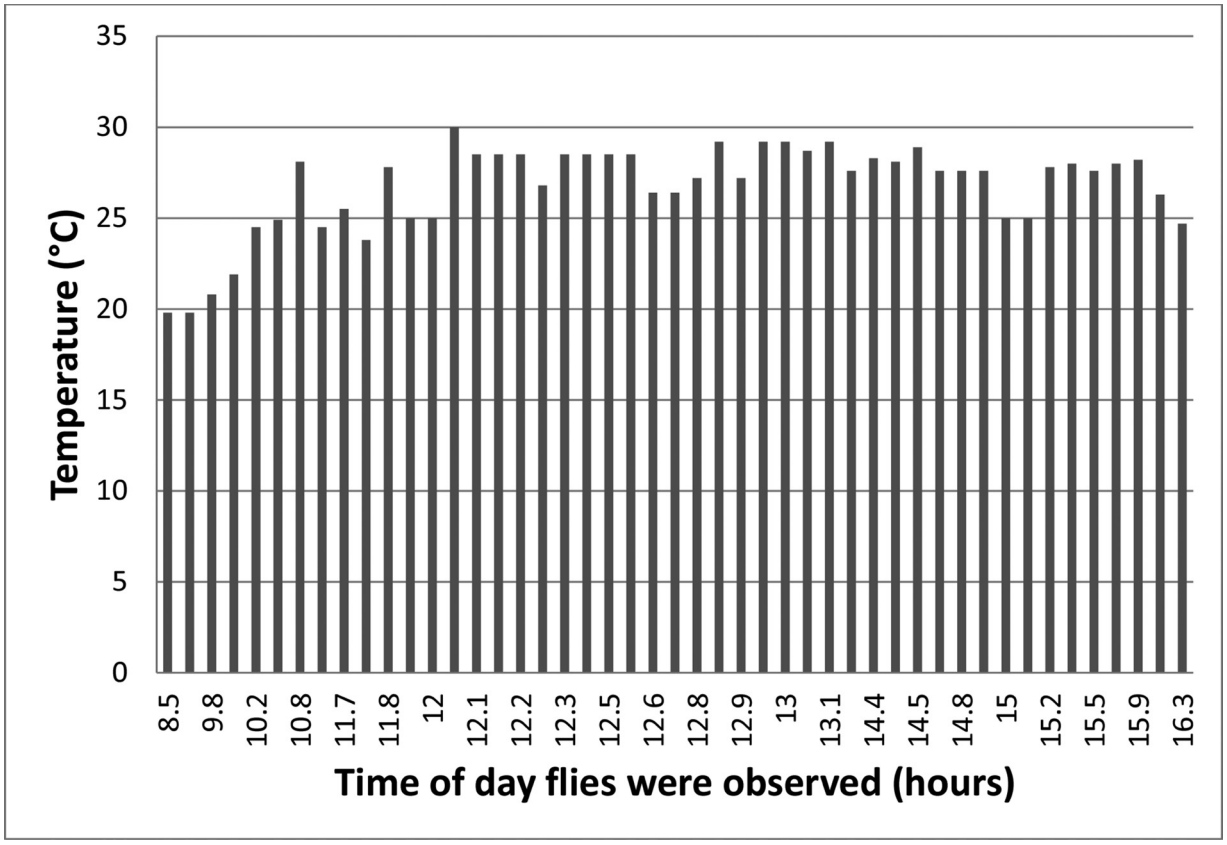

Fig. 3. Temperature and time of day at which individual chloropid flies were observed on inflorescences of *Genoplesium littorale* (12–14 March 2013).

All advanced buds had intact pollinia in the anthers and no pollen on the stigma, indicating that no pre anthesis self-pollination had occurred (Table 5). No case was found of post anthesis pollinia breakdown and spillage of pollen from anthers onto the stigma. Nor was there any evidence of growth of pollen tubes through the back of the stigma, or outgrowths of the stigma meeting the pollinia, that might also indicate self-pollination. In addition, in all but one flower out of 54, swelling of ovaries was only present where pollen was also observed on the stigma, ruling out apomixis.

Table 5. Numbers and percentages of flowers from which pollinaria had been removed and stigmas pollinated, aggregated from ten inflorescences collected in 2012 (three flowers) and 2013 (seven flowers).

Flower status	No. of flowers	Pollinaria removal		Pollen on stigma	
		No.	%	No.	%
Advanced buds	8	0	0.0	0	0.0
Anthesis	50	24	48.0	6	12.0
Post anthesis-ovary unswollen	29	18	62.1	3	10.3
Post anthesis-ovary swollen	54	41	75.9	53	98.1

Examination of 29 single flowers for potential brood sites found no evidence of egg laying by chloropids. Similarly, no insect eggs were observed on any of the 141 flowers examined for pollinaria removal and pollen deposition.

Plant survival: The pollination success of tagged *G. littorale* plants was determined on 23 April 2013. Of the remaining 131 labelled plants, only 60, or less than half the sample (45.8%) remained in a viable condition (Table 6). A quarter of the plants (24.4%) were lost to herbivores, which removed the inflorescences and varying proportions of the stem. Another quarter (25.2%) of the plants was missing altogether, i.e. no above ground parts remained. It is likely that most of these were also lost to herbivory, suggesting that up to half the plants were consumed before they could produce seed. A small proportion of plants (3.8%) had shrivelled inflorescences for unknown reasons.

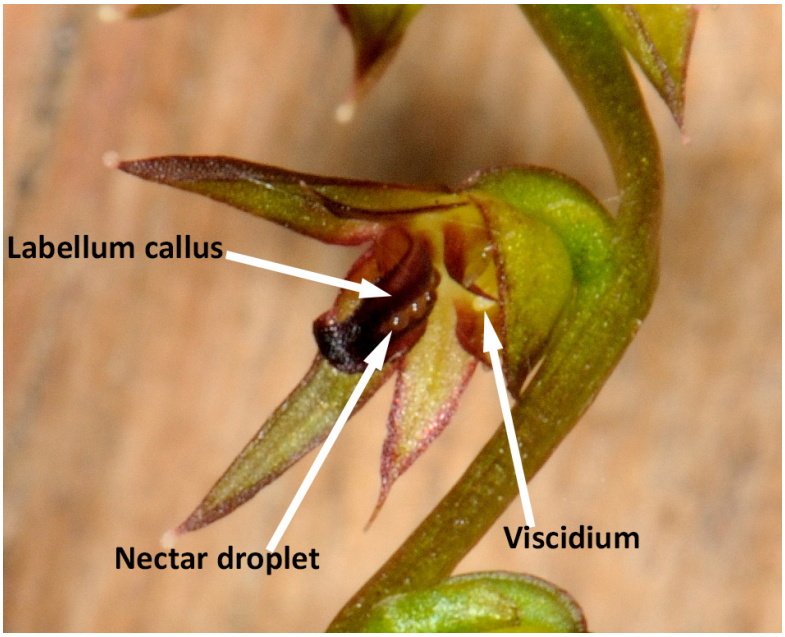

Fig. 4. Nectar droplets in the groove of the *Genoplesium littorale* labellum callus. In walking up the labellum of the flower towards the base while consuming nectar, the pollinator contacts the viscidium which adheres to its thorax.

Table 6. Loss of *Genoplesium littorale* plants to herbivory across four sub-populations.

Group	Herbivory		Missing		Shrivelled		Extant		Total
	No.	%	No.	%	No.	%	No.	%	
A	4	12.5	6	18.8	1	3.1	21	65.6	32
B	10	52.6	6	31.6	1	5.3	2	10.5	19
C	13	25.5	18	35.3	2	3.9	18	35.3	51
D	5	17.2	3	10.3	1	3.4	19	65.5	29
Total	32	24.4	33	25.2	5	3.8	60	45.8	131

Levels of herbivory were much higher in groups B and C than in groups A and D (Table 6). The differences in herbivory (partially consumed plus missing plants) between groups is highly significant ($P<0.001$, chi square test).

Fruit set: The overall proportion of flowers setting fruit on the surviving 60 plants averaged 42.6% across the whole study area (Table 7). Fruit development was highest in groups B and D, 57.9 and 55.0%, respectively, although there were only 2 extant plants on area B (Table 7). The percentage of fruit set in groups A and C was almost half that in groups B and D. The percentage of fruit set differed significantly ($P<0.05$) between subpopulations A and D, and C and D (Table 8).

Table 7. Numbers of surviving plants, viable flowers and fruit capsules in four sub-populations of *Genoplesium littorale* (Analysis of Variance showed significant differences among the mean percentages of fruit capsules between sub-populations, $P<0.01$).

Group	No. of extant plants	Total viable flowers	Mean flowers/plant	Fruit capsules		
				Total	Mean/plant	Mean percent
A	21	215	10.2	65	3.1	27.1
B	2	43	21.5	25	12.5	57.9
C	18	212	11.8	74	4.1	34.1
D	19	225	11.8	132	6.9	55.0
Total	60	695	11.6	296	4.9	42.6

Table 8. Least Significance Difference (LS D) tests ($P<0.05$) for percentages of fruit capsules among the pairs of sub-population means in Table 7. (The Least Significant Difference for each comparison is in the upper right and the significant differences are in the lower left.)

Sub-populations	A	B	C	D
A	-	16.4095	16.1964	37.8544
B	no	-	16.8254	38.1277
C	yes	yes	-	38.0272
D	no	no	no	-

Discussion

Pollinators: In agreement with previous observations on other species of *Genoplesium* (Bower, 2001a), this study found the pollinators of *G. littorale* are five putative species of small flies in the family Chloropidae. This family is widespread, but poorly known in Australia (Colless and McAlpine 1991). The chloropid pollinators of *G. littorale* are common, and result in high pollination percentages on some plants. High pollination levels are likely when inflorescences at the peak of their attractiveness coincide with warm days above 25°C, which favour chloropid activity. Such conditions occurred when this study was undertaken in mid-March 2013 and 2014 (Fig. 3). Over three days of collection in 2013, in excess of 50 observations of pollinators on plants were made, on occasions with multiple individuals of two different chloropid species on the same plant. Similarly in 2014, 33 chloropids in the same four putative *Conioscinella* species as in 2013 were collected over four days and many more were observed. *G. littorale* is stimulated to flower by high rainfall in late summer and early autumn, which may also stimulate emergence of adult chloropids, thereby achieving synchrony between plant and pollinator.

Pollinator specificity: The specific relationship between outcrossing *Genoplesium* species and pollinators in the related fly families Chloropidae and Milichiidae is unusual, especially for orchids providing nectar rewards. Generally, when nectar is available, it is exploited by a diverse range of insects from several insect orders, e.g. Hymenoptera, Diptera, Coleoptera and Lepidoptera, and many families and genera within them. This is true of the nectar-rewarding orchid genus *Prasophyllum*, that is closely related to *Genoplesium* (Bower 2001b). However, although there is a specific relationship between *Genoplesium* and the families Chloropidae and Milichiidae, pollinator specificity is lacking at the species level in *G. littorale*, which attracts multiple chloropid species as pollinators and accordingly is oligophilous. *G. littorale* is only the second *Genoplesium* species clearly shown to attract multiple chloropid or milichiid fly species. Bower (2001a) reported that flies collected on *G.* aff. *rufa* by A. Logan included two chloropid species and a milichiid, although it was not determined if all were capable of effecting pollination.

Pollination mechanism of *Genoplesium littorale*: The high levels of pollinaria removal (71% in post anthesis flowers) and the presence of pollen on the stigmas of 98% of flowers developing fruit indicates *G. littorale* is dependent on its chloropid vectors for pollen transfer (Table 5). Observations of floral morphology did not support the existence of autogamy or apomixy in *G. littorale*. If *G. littorale* was obligately autogamous all flowers would retain the pollinarium as a prerequisite for autogamy. However, the data show the pollinarium is always removed as a whole, and was removed from over 70% of flowers, which clearly precludes autogamy in those flowers. If the flowers were facultatively autogamous, those that retain their pollinarium and are not pollinated by vector transfer would develop fruit. All 15 such flowers in the inflorescence examinations failed to develop fruit. In any event, detailed examination of 141 flowers found no mechanism for self-pollination. Finally, the percentages of flowers setting fruit are well below the high levels expected for autogamy or apomixy in orchids (Neiland and Wilcock 1998). It is concluded that *G. littorale* is an obligately outcrossing species dependent on pollen vectors in the family Chloropidae.

Pollination strategy: The presence of nectar was observed in *G. littorale* (Fig. 4) in this study confirming nectar reward. However, the absence of nectar on five out of six flowers photographed in the field suggests that nectar is quickly removed by pollinators. The presence of nectar on 55% of flowers stored for one or two days in refrigerated vials supports the conclusion that nectar is rapidly removed by insects in the field. The nature of the nectar was not investigated in this study. Nectar composition may provide important clues to the specific attraction of chloropids to *Genoplesium*.

The biological basis of the specific relationship between *Genoplesium* and chloropids and/or milichiids is unknown. It has generally been assumed that *Genoplesium* attracts chloropids with food odours and a nectar reward (Bower 2001a). However, the specificity of the attraction suggests that *Genoplesium* flowers may be offering a specialised reward uniquely attractive to chloropids and milichiids. Detailed searches of 29 flowers and examination of 141 others revealed no oviposition by chloropids, ruling out the brood site mimicry hypothesis. Similarly, the lack of strong dung or carrion-like odours suggests *G. littorale* is unlikely to be sapromyophilous.

An intriguing possibility is that outcrossing *Genoplesium* species may be another example of 'kleptomyiophily' so far known only from *Aristolochia rotunda*, which is pollinated mainly by three species of female chloropids (Oelschlägel *et al*. 2014). Similar to *A. rotunda* the dominant pollinators in this study were female chloropids, tentatively assigned to five species. In both *A rotunda* and *G. littorale* a small proportion of the pollinators were male chloropids of the same species. However, by contrast with *A. rotunda*, which provides no reward for its pollinators (Oelschlägel *et al*. 2014), *G. littorale*, and other *Genoplesium* species, produce nectar upon which pollinators feed. Clearly, much remains to be learnt about the attraction of chloropids to *Genoplesium* and the nature of the rewards offered.

Reproductive success: In the 2013 season, between 27 and 58% of flowers in the four sub-populations of *G. littorale* developed fruit capsules, mean 44%, which indicates relatively high reproductive success (Table 7). The pollination levels found in this study compare favourably with those recorded in orchids generally, which are often very low (Gill 1989, Neiland and Wilcock 1998, Tremblay *et al*. 2005). The mean fruit to flower ratio of 44% in *G. littorale* is similar to that typically found in orchids providing nectar rewards and much higher than the average of deceptive species (Neiland and Wilcock 1998, Tremblay *et al.* 2005). For 30 nectar-rewarding orchid species, mean fruit set was 50.8% (range 12.5 to 96.0%), whereas 73 deceptive species averaged 22.2% (range 0 to 69.5%) fruit set (Neiland Wilcock 1998). Equivalent fruit set data from Tremblay *et al*. (2005) for temperate species only is: 58% rewarding species, 41.8% (range 0 to 96.6%); 76 deceptive species, 28.4% (range 1 to 69.5%).

Herbivory: Results showed that less than half the inflorescences survived from flowering to fruit capsule maturity. Most were lost to herbivory. Losses were greatest in areas with dense shrub cover and least in open areas, or counter-intuitively, close to a main road, firebreak and residences, where adverse influences due to

humans might be expected. Herbivory as a threatening process for rare and endangered Australian orchids is a significant issue (Petit and Dickson 2005, Faast and Facelli 2009). The high loss of *G. littorale* plants to herbivores is of concern for this critically endangered species within its highly restricted distribution. Consideration should be given to more intensive management of this population to minimise herbivory, particularly herbivore proof fencing.

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