The ecology, evolution and persistence of an obligate, one-on-one mutualism

BY

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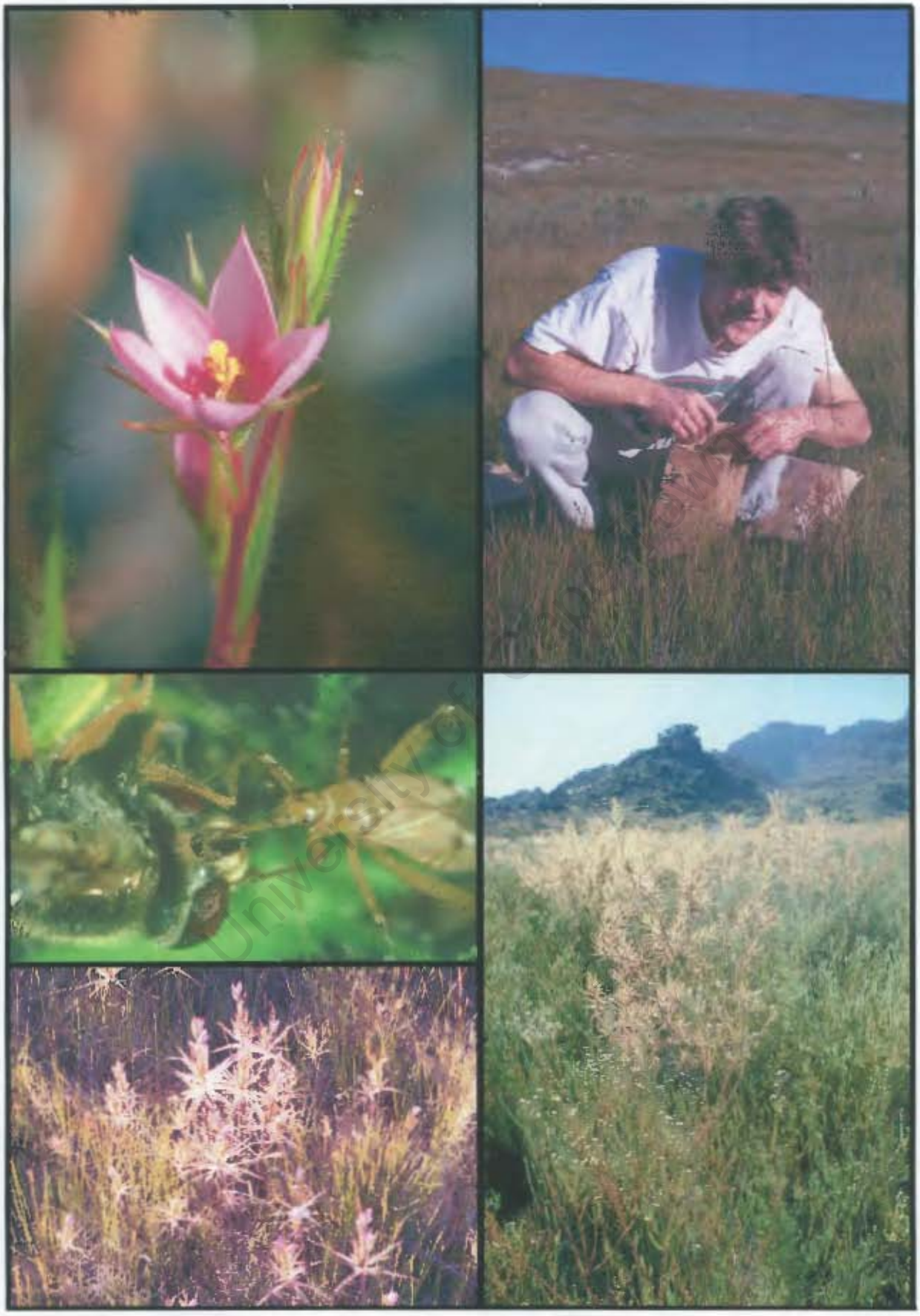
Statement

The ideas, thought and writing of this study were entirely my own except in the instances mentioned below – also see acknowledgements at the start of the thesis and at the end of certain chapters.

Nicola Bergh conceived of the original idea for chapter 3 although I conducted the execution and subsequent work on this chapter. Chapters 1, 2 and 5 were adapted from papers (Anderson *et al.*, In Press; Anderson and Midgley, In Press; Anderson and Midgley 2002) co-authored by my supervisors Jeremy Midgley and Barbara Stewart. Jeremy's ideas were especially important in the methods of chapter 5. Isabelle Olivieri conceived of some of the original ideas used in chapter 9. All my supervisors: Jeremy Midgley, Isabelle Olivieri and Barbara Stewart helped to improve existing manuscripts through a process of discussion and suggestion.

Top left. *Roridula gorgonias* in flower (Photo-Amy Spriggs). **Top right**. The author crouches amongst *Roridula gorgonias* seedlings (Photo-Amrei von Hase). **Middle left**. The mutualism plays itself out: *Pameridea* sucks the juices from a fly before defecating on the leaves of *Roridula*. **Bottom right**. A *Roridula dentata* population glistens in the late afternoon sunlight (Photo-Amrei von Hase). **Bottom left**. Beauty belies a lethal carpet of young *Roridula gorgonias* plants (Photo-Amrei von Hase).



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"Obligate one-to-one mutualisms between species pairs are rare in practice and anomalous in theory." Howe1984

ABSTRACT

Some of the most celebrated examples of coevolution are found amongst obligate, one-on-one mutualisms (e.g. fig and yucca pollination systems). Although obligate, one-on-one mutualisms may be common among intimate, endosymbiotic organisms, they are apparently uncommon between free-living or non-symbiotic organisms (Howe 1984). Many theories have been postulated to explain the rarity of obligate, one-on-one mutualisms but due to the limited number of examples, it is very difficult to test these theories. The aims of his thesis were to examine the mutualism between an insect catching plant (*Roridula*) and a closely associated hemipteran to determine whether current theories fit this system. More specifically, I determine: 1) whether *Roridula* is obligately dependent on *Pameridea*. 2) Whether the exploitational nature of mutualism causes conflict in this system. 3) Whether exploiters significantly affect the outcome of the mutualism. 4) What effect habitat fragmentation has on the genetic variability of both species. 5) The major processes driving speciation and the spatial scale at which adaptation occurs in this system.

This thesis is broadly broken up into five sections, each one answering one of the above questions (although there is significant overlap of sections). Each chapter represents a "stand-alone" document, which can be submitted as a paper. Hence there is some repetition in the introductory sections of each chapter. In chapters 1-3, I deal with the reward system offered by hemipterans. In chapter 1, I show that *Pameridea* is a very important pollinator and that they are responsible for a large proportion of seed set in *Roridula gorgonias* and *Roridula dentata* (even though most of this seed is selfed, i.e. facilitated selfing). The remainder of the seed set is the result of self-pollination and additional pollinators play a minor role in the quantity of seeds set. The presence of hemipterans significantly enhances reproductive output in *Roridula*. In addition, this is one of the few cases where facilitated selfing is shown to be adaptively significant (facilitated selfing is thought to decrease plant fitness in other species and is hence non-adaptive). Chapter 2 determines the contribution of insect derived nitrogen in *Roridula gorgonias* using natural abundance levels of $\delta^{15}N$. More than 70% of plant nitrogen in *Roridula gorgonias* is derived from insects, which is comparable to levels found in the most insect dependent plants. I propose an extension of the definition of carnivory, which includes semi-carnivorous plants that indirectly absorb nutrients using mutualisms that are obligate and species-specific. In chapter 3, I examine the mechanism of nitrogen absorption in *Roridula* using the dye, neutral red. Aqueous compounds are readily absorbed via *Roridula* leaves. In contrast, non-carnivorous plants are not

able to rapidly absorb neutral red through their leaves. Examination using TEM revealed that the cuticle of *Roridula* is exceptionally thin and frequently discontinuous. This exposes the permeable cell wall elements to the outside of the leaf, which enables the rapid absorption of aqueous compounds from the leaf surface. A discontinuous cuticle is possibly another adaptation to the carnivorous syndrome

Chapter 4: One of the constraints on the evolution of obligate, one-on-one mutualisms is that so-called mutualisms are often antagonistic under certain conditions. I determine what effect different hemipteran densities have on the growth rates of *Roridula* plants in the laboratory. *Roridula* has very poor growth rates in the absence of hemipterans. Under intermediate hemipteran densities, *Roridula* has good growth rates suggesting that the presence of hemipterans is crucial for nitrogen uptake. However *Roridula* has very poor growth rates when hemipteran densities are high due to the fact that *Pameridea* also sucks sap from *Roridula*. These observations highlight the exploitative nature of mutualisms and suggest an antagonistic origin for the mutualism. Antagonism may be an important precursor to obligate, one-on-one mutualisms because antagonism often facilitates specialization and specificity.

Another factor, which may constrain the evolution of obligate, one-on-one mutualisms, is the tendency for mutualisms to attract additional species, which have detrimental effects on the mutualism. In chapter 5, I examine the effects of specialist spiders on the functioning of the mutualism. Spiders on *Roridula dentata* prey upon and compete with hemipterans for food, lowering their densities. Populations with high spider densities have low hemipteran densities and consequently a much lower proportion of plant nitrogen is derived from insects (as little as 40%). Although spiders lower hemipteran densities significantly, hemipteran densities never reach low enough levels to make indirect carnivory "unprofitable". Hence, the spider invasion is unlikely to cause mutualism breakdown at its observed levels. However, it is possible that spiders could decrease the rate at which specialization takes place by weakening coevolutionary selective pressures. Spiders may also stabilize the mutualism between *Pameridea* and *Roridula* by keeping *Pameridea* densities at optimal levels. In chapter 6, I use present distribution patterns of *Roridula* and associated fauna to determine the relative ages of *Roridula*-arthropod associations. Hemipteran distributions are correlated exactly with *Roridula* distributions and suggest that the association between two genera is relatively ancient. In contrast two specialist spiders have distributions that only occupy small parts of *Roridula dentata*'s distribution range. I postulate that their history of association is shorter than that of *Roridula-Pameridea*. The absence of spiders from this mutualism during its early stages may have provided the necessary stability for specialization to evolve between *Roridula* and *Pameridea*.

The fragmentation of mutualist host populations may in turn cause fragmentation and genetic erosion of its partner's populations. Chapter 7 uses allozyme electrophoresis to examine the genetic variability of *Roridula* and *Pameridea*. Genetic variability in *Roridula* is low, suggesting that fragmentation and bottlenecks have caused considerable genetic erosion in this genus. In contrast, genetic variability in *Pameridea* is high, reflecting the ability of hemipterans to move between fragmented *Roridula* populations and function as a metapopulation. Fragmentation of *Roridula* has had little effect of the genetic variability of *Pameridea*. Nevertheless conservation efforts must ensure that sufficient *Roridula* populations survive so that the metapopulation structure of *Pameridea* persists.

Chapters 8 and 9 examine the processes driving speciation and the spatial scale at which adaptation occurs in this system. In chapter 8, I compared the genetic structures of *Roridula* and *Pameridea* using allozyme electrophoresis. Gene flow of both *Pameridea* and *Roridula* are restricted and should enable host-race formation to occur at the population or regional level. However distribution patterns and dispersal abilities of *Roridula* and *Pameridea* have overriding effects on the genetic structures of these species. Consequently allopatric speciation is likely to be the major speciation mechanism in this system and can be driven by genetic drift. Adaptive deme formation (~~to~~ intrinsic factors) is likely to play a secondary role in the speciation process. This is in concordance with the geographic mosaic theory (Thompson 1994), which predicts that the geographic structures of closely associated organisms have a major effect on the coevolutionary process. In chapter 9, host choice and "reciprocal transplant" experiments were used to determine the specificity of *Pameridea* and to assess whether local adaptation has taken place. *Pameridea* fails to reproduce successfully on non-carnivorous plants, confirming observations that *Pameridea* seems to be host-specific and therefore obligately associated with *Roridula*. Nevertheless *Pameridea* is able to survive for long periods on non-carnivorous plants by sucking plant sap. This adaptation may be necessary to facilitate movement between *Roridula* populations. *Pameridea* has preferences (interspecific) for its host species of *Roridula* over non-host species of *Roridula*. However, *Pameridea* show no preference towards plants from natal populations over plants from non-natal populations (intraspecific). Nor does fitness vary on natal versus non-natal populations of the same species. I attribute this to the fact that phenotypic differences at the sub-specific level are probably not large enough to cause adaptive deme formation in this system. The lack of extensive local adaptive deme formation in this system may explain the paucity of species in the *Roridula-Pameridea* mutualism.

CONTENTS

"Mutualism may be everywhere, but its existence remains practically unproven."
Boucher 1982

INTRODUCTION

The study of mutualisms

Mutualisms have been implicated in phenomena as fundamental as the evolution of eukaryotic cells (Margulis and Fester 1991) and the radiation of angiosperms (Regal 1977), and they are found in every ecosystem and every organismal kingdom (Boucher 1985). Their ubiquity ensured that mutualisms were perceived as far back as ancient Greek times (Boucher 1985) and in 1875, the term was used to describe interspecific interactions that benefit both species (Boucher *et al.* 1982). This new term allowed biologists to distinguish mutualism from antagonism and commensalism.

Despite the early beginnings and great importance of mutualism study, many feel that the subject has been grossly neglected (e.g. Abrams 1987, Boucher 1985, Keddy 1989), to the extent that some of the most basic terminology used to describe mutualisms remains unsettled (Lewis 1985). However, a study of primary literature (nine journals) revealed that about 22 % of all articles on species interactions (1986-1990) investigated mutualisms (Bronstein 1994a). It seems that although there is a great volume of literature regarding mutualisms, few studies did more than just describe them and even fewer quantified the reciprocal effects of the mutualisms. Instead, researchers in the past have generally treated mutualisms as a life history trait of either one of the partners and hence the resulting picture is very one-sided and few evolutionary questions have been examined in depth (Bronstein 1994a). It is only recently that studies have started to focus on the ecological and evolutionary patterns in mutualisms (Bronstein 1994b). As a result, we know very little about how mutualisms evolve, function or persist. In fact, few studies have even demonstrated that mutualisms can increase the fecundity or growth of both interacting species.

One of the most interesting observations to emerge from the plethora of descriptive data is that highly species specific, non-symbiotic mutualisms are rare (Hoeksema and Bruna 2000). Although mutualisms are ubiquitous, they tend not to be host-specific (Thompson 1994). For example, plants are seldom pollinated by a single, species-specific pollinator. Instead, most plants are pollinated by several pollinators and most pollinators pollinate a wide variety of plants (Waser *et al.* 1996). Notable exceptions to this rule include deceptive mimics such as the many orchid species that are pollinated by a single species of male wasp, which attempts to copulate with the flowers (e.g. Steiner *et al.* 1994). However, these relationships are not mutualistic because the wasps receive no rewards for their efforts.

In contrast, fig and yucca pollination systems are two of the most well known obligate, species-specific mutualisms and both are founded upon antagonistic principals (Davis 1967, Addicott *et al.* 1990): in both of these systems pollinators are also seed parasites. Thus, pollination comes at the cost of fertilised ovules. If pollinators in these systems parasitize too many ovules, then the net outcome for plants may be detrimental and not beneficial. Seed parasitism was also found to be the price for pollination in another highly species-specific, obligate mutualism between globe flowers which are pollinated by four closely related fly species (Pellmyr 1989). Interestingly, species specificity in parasitism seems to be very common and is perhaps the norm (Thompson 1994), possibly relating to the fact that parasites often have to overcome unique host defences. This may explain why all of the highly specific mutualisms (figs, yuccas, globeflowers) are thought to have evolved from previously antagonistic relationships (Davis 1967, Pellmyr 1989, Addicott *et al.* 1990).

In conventional pollination mutualisms, plants usually supply a pollen or nectar reward for the pollinators. However, elements of antagonism can even be observed in conventional pollination systems when pollinators take rewards without actually pollinating the flower (e.g. Colwell *et al.* 1974; Inouye 1983). These examples of pollination mutualisms highlight the way mutualisms are presently thought of: mutualisms are actually reciprocal exploitations where each partner attempts to secure as large a reward as possible from the other (Axelrod and Hamilton 1981, Soberon Mainero and Martinez del Rio 1985, Bull and Rice 1991, Pellmyr and Huth 1994, Herre and West 1997, Doebeli and Knowlton 1998). Despite this conflict of interests, the net outcome of mutualisms is usually beneficial and not detrimental to both partners (i.e. they both receive rewards). However, the net outcome of so-called "mutualisms" is seldom stable in space and time (Bronstein 1994b). Instead, the outcomes may oscillate from being antagonistic to mutualistic in different parts of the geographic ranges of interacting species, or with changing biotic and abiotic factors (Thompson 1994). Hence the net outcomes of mutualisms (beneficial or detrimental) are inherently "conditional" or variable and this is may be one of the major constraints on the evolution of obligate, one-on-one mutualisms. Bronstein (1994b) predicted that the outcomes of facultative mutualisms should be more conditional than the outcomes of more obligate mutualisms. Present data in the form of ant-plant mutualisms seem to support this prediction. Ant-plant mutualists are seldom species-specific and obligate for both partners and over half of the studies show no apparent benefits to the plants (Becerra and Veneble 1989). However in obligate, species-specific yucca mutualisms, plants abort fruits, which have been heavily parasitized, and in this way they impose selective pressures on the moths to be less detrimental. This results in a relationship, which is generally

mutualistic and where the net outcomes are more predictably positive (Pellmyr and Huth 1994). In globeflowers, fly density seldom reaches antagonistic levels and hence the system is mutualistic over a wide range of circumstances, possibly allowing a high degree of specialization and obligacy to evolve (Pellmyr 1989).

The evolution of species-specific mutualisms is also constrained by several other sources of conditionality: For example, mutualistic interactions tend to attract additional species (exploiters) that take advantage of the resources meant for mutualists but do not reciprocate by returning "commodities" (Thompson 1994, Bronstein 2001). These additional species may affect existing mutualisms adversely by competing for resources without adding any benefits to the mutualist partners (Bronstein 2001). Exploiters may destabilize mutualisms by causing unpredictability in their reward systems (Bronstein 1994b, Bronstein 2001). For example, birds or bees that are not adapted to pollinate certain long tubed flowers may steal nectar by puncturing or chewing through the corolla tube without pollinating the flowers (Inouye 1983). Because the invasion of mutualisms by cheaters may cause variation in the reward quality of mutualisms, cheaters such as nectar thieves may cause potential pollinators to seek flowers with more reliable rewards (Soberon Mainero and Martinez del Rio 1985). The result may be mutualism collapse, which could trigger the extinction of obligate partners (Boucher *et al.* 1982). Although, there is no empirical evidence to show that exploiters can cause the collapse of established mutualisms, they may halt the evolution of specificity and specialization of developing mutualisms. Additional species may be especially detrimental to specialization in mutualisms if they prey upon mutualists, affecting their numbers and hence the benefits to other mutualist partners. Even if additional species do not affect mutualisms directly, diverse faunas may dilute strong directional selection pressures, resulting in a selective regime that is too diffuse for tight coevolution between any two species (Hoeksema and Bruna 2000).

The evolution and maintenance of specialist mutualisms is thus constrained by 1) the exploitative nature of mutualisms, 2) the tendency for existing mutualisms to attract new species (cheaters, predators), 3) The inability of hosts to exclude exploiters, which results in unpredictability of the visitor assemblage, 4) rewards, and 5) diffuse selective regimes. Finally, 6) mutualist densities may be unpredictable and fluctuating in space and time. In order to escape the detrimental effects of diverse faunas, close one-on-one mutualisms are sometimes thought to evolve in environments with low species diversity.

Organisms in obligate, symbiotic relationships may also have an increased risk of extinction due to the frailties of their own partners. This may further increase the risk of mutualism breakdown over evolutionary time, lowering the chances of

obligacy evolving. For example, habitat fragmentation may cause reductions in population size and genetic erosion of a single partner (host). Reductions in host population size may in turn cause reductions in population size of associated mutualists, with cascade effects on genetic diversity. To my knowledge, the comparative genetic diversity of mutualistic species has never been investigated, even though it may have strong implications on how mutualisms are maintained over time and on how best to conserve mutualisms and the species involved.

In addition, gene flow and the resulting genetic structure of mutualists has a great influence on the ability of organisms to coevolve: For example, restricted gene flow is thought to enhance the potential for local adaptation (Slatkin 1987), although Mopper (1996) found no correlation between parasite mobility and local adaptation. Too little gene flow may also constrain local adaptation by depleting genetic variability (Gandon *et al.* 1996) and too much geneflow may swamp local adaptations (Slatkin 1987). In most studies, only the genetic structure of parasites has been studied, frequently showing genetic subdivision and the existence of host races (McPheron *et al.* 1988, Feder *et al.* 1988, Rank 1992). It is thought that parasites may be able to adapt at the microgeographic scale to differing phenotypes of individual host plants or host populations and thus host race formation may have adaptive significance (Mopper 1996). One way of inferring that local adaptation has taken place is by showing correlated genetic structures of parasites and their hosts (this assumes that phenotypic differences are correlated with genotypic differences, e.g. Mulvey *et al.* 1991; Dybdahl and Lively 1996). However correlated genetic structures may also be due to similar patterns of dispersal and thus be caused by genetic drift. Nevertheless, correlated genetic structures may enhance the potential for coadaptation, irrespective of the cause. Examination of genetic structure may also indicate at what scale coevolution is most likely to take place (i.e. at the level of individual hosts, populations, regional or species level). Despite the importance of comparing the genetic structures of closely associated organisms, very few studies have overlaid host-parasite genetic structures and no studies have done so for mutualisms. Another theory to emerge recently is that the geographic distributions of closely associated organisms have a profound on the coevolutionary process (Thompson 1994) and thus an organism's genetic structure. Geographic mosaics of different interacting organisms may cause coevolutionary hot spots and cold spots to develop.

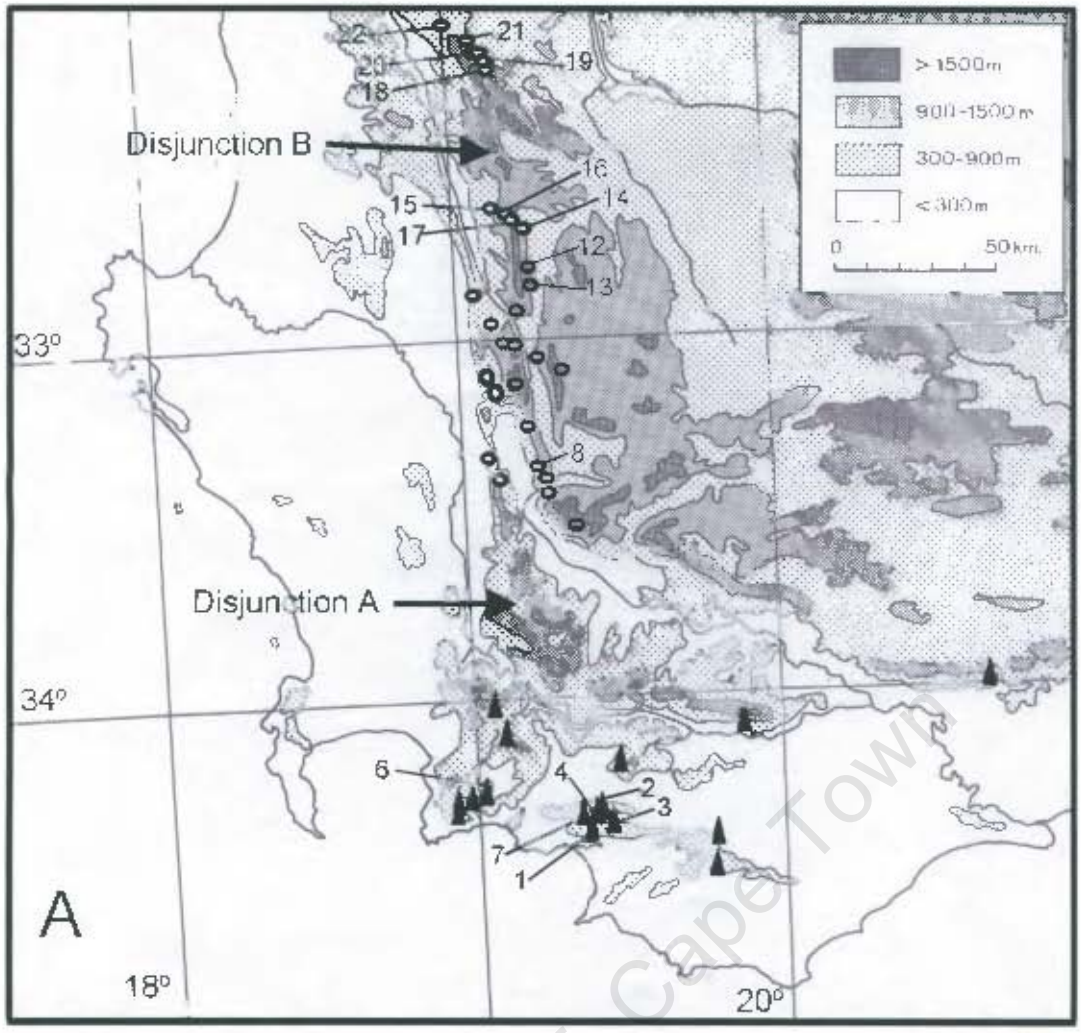
The Roridula-Pameridea *mutualism*

To answer some of the pressing questions regarding the evolution and persistence of mutualisms I chose to study the interaction between the semi-carnivorous host plant *Roridula* and its hemipteran mutualist, *Pameridea*. The plant family Roridulaceae is a monophyletic group consisting of a single genus and two species (Obermeyer 1970) endemic to South Africa. Based on molecular phylogeny, the genera most closely related to *Roridula* are from the family Sarraceniaceae and include the carnivorous genera *Darlingtonia*, *Heliamphora* and *Sarracenia* (Bayer *et al.* 1996, Conran and Dowd 1993, Chase *et al.* 1993), none of which occur in South Africa. The taxonomic and geographic isolation from its closest relatives suggests that *Roridula* is a paleoendemic genus (Linder *et al.* 1992). Paleoendemics are systematically isolated taxa with relatively ancient origins (Stebbins and Major 1965). They are often ecological specialists and are perhaps "on the way to extinction" (Stebbins and Major 1965). Their distribution ranges are relictual and their narrow, fragmented distributions are the result of a formally more extensive geographic distribution (Stebbins and Major 1965). The two *Roridula* species (*R. gorgonias* and *R. dentata*) are geographically separated (Obermeyer 1970, Carlquist 1976) with *Roridula gorgonias* occupying the southern mountains of the Riviersonderend, Perdeberge and Koggelberg and Langeberg (Fig. 1). The two species are separated by approximately 70 km (disjunction A, Fig. 1). *Roridula dentata* has a disjunct distribution starting in the Ceres region and following the Grootwinterhoek Mountains and Kouebokkeveld mountains to about Citrusdal. From Citrusdal to the Southern Cedarberg, there seem to be no records of *Roridula* populations (disjunction B, Fig. 1). The plants of both species also tend to be found in isolated and very discrete populations. Populations can vary in size from between 5 and 2000 individuals. Usually plants are fairly densely packed, frequently touching one another. Old *Roridula dentata* populations (> 10 years) are sometimes sparse and plants may be separated by up to 5 metres. *Roridula* is closely associated with moist habitats and the shrinking of these habitats with changing climatic conditions may have caused the disjunct distributions observed for the genus. *Roridula gorgonias* occurs in peat seeps and marshy areas with permanent surface water (Carlquist 1976, pers. obs.), similar to the closely related Sarraceniaceae. *Roridula dentata* can be found on sandy "vlakte" (flats) that are drier than *R. gorgonias* localities in summer although underground water seems plentiful (Carlquist 1976, pers. obs.). In winter these areas are very wet (Carlquist 1976, pers. obs.).

The leaves of *Roridula* are covered by hairs, each one supporting a sticky, resinous droplet. In this respect *Roridula* looks similar to *Drosera* except that *Roridula* plants can grow much larger (up to 2m tall) and they have woody stems. *Roridula* are also

Figure 1.

1. Map of *Roridula* distribution where labelled populations represent populations studied by the author, circles represent *R. dentata* populations and triangles represent *R. gorgonias* populations. Unlabelled points are approximate positions of populations known only by herbarium records or populations, which the author was unable to visit.



more sticky than *Drosera* to touch. The droplets catch large numbers of insect prey but unlike *Drosera*, *Roridula* has no digestive enzymes to digest their prey. Despite the absence of digestive enzymes, Ellis and Midgley (1996) discovered that *Roridula gorgonias* plants were still able to assimilate insect nitrogen within 72 hours of the insects being captured. The efficiency of this nitrogen assimilation hinges on the presence of a small hemipteran (*Pameridea roridulae*) that inhabits *R. gorgonias* plants in large numbers. This insect consumes the prey caught by *R. gorgonias* and defecates on the plants' leaves. It is thought that the faecal nitrogen is then taken up by the leaf. Ellis and Midgley (1996) used flies enriched with ^{15}N to examine the uptake of nitrogen into the plant. They found that very strong traces of ^{15}N were found in plants with hemipterans but comparatively weak traces of ^{15}N were found in plants where *Pameridea* was absent. In support of the foliar absorption theory, *Roridula* is known to have a very thin leaf cuticle (Bruce 1907), which may make foliar absorption possible. *Roridula* also has a very poorly developed root system (Carlquist 1976) possibly due to the fact that they do not need to absorb nutrients. Only one other study has examined *Roridula* nutrition (Midgley and Stock 1998), and they analysed natural abundance levels of ^{15}N in *R. gorgonias* and co-occurring plants. Carnivorous plants are expected to be ^{15}N-enriched compared to co-occurring reference plants because $\delta^{15}N$ values increase by 3-5 ‰ per trophic level (fractionation, Schulze *et al.* 1991). Midgley and Stock (1998) found that *R. gorgonias* plants had higher $\delta^{15}N$ values than non-carnivorous plants and carnivorous *Drosera*, confirming that *Roridula* also obtains a substantial proportion of their nitrogen from insects. They also found that *P. roridulae* were not normally associated with small *R. gorgonias* seedlings. These seedlings had much lower $\delta^{15}N$ values than co-occurring adults, suggesting that the presence of *P. roridulae* is crucial for the assimilation of insect nitrogen. *Roridula dentata* is also associated with a hemipteran (*Pameridea marlothi*) which possibly performs a similar nutritional role to its sister species. The two *Pameridea* species are the only two species in the genus and each is obligately associated with a single *Roridula* species, being found nowhere else (Dolling and Palmer 1991). *Pameridea* belong to the family Miridae that is largely phytophagous and often very species-specific or monophagous (Schuh 1995).

In addition to *Pameridea*, *Roridula dentata* also has associations with several other species of arthropod whose nutritional roles are unknown. These arthropods include three species of spider (*Synaema marlothi*, Thompsidae; *Peucetia nicolae*, Oxyopidae and an unidentified Araneidae) and one hemipteran *Sphedanolestes* sp. In contrast *R. gorgonias* populations are not routinely associated with any arthropods other than *Pameridea*.

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PART 1

OBLIGACY IN *RORIDULA*

1 Facilitated selfing offers reproductive assurance: a mutualism between a hemipteran and carnivorous plant

ABSTRACT

Controversy has surrounded the identity of the pollinator of the near carnivorous plant genus *Roridula*. Hemipterans, which have a digestive mutualism with *Roridula*, have been implicated in pollination but flowers show adaptations to hymenopteran pollination. Through floral manipulations, field observation and allozyme electrophoresis, I deduce that hemipterans are the primary pollinators of *Roridula* as seed set is significantly reduced when they are excluded from the flowers. The nutritional mutualism extends further than a digestive function. Although bees still pollinate *Roridula* on very rare occasions, their exclusion does not affect mean seed set. The complicated floral structures that occur in *Roridula* are most likely adaptations for maximising outcrossing by bees, although allozyme electrophoresis results suggest that almost all seeds are selfed. I hypothesise that resident hemipterans facilitate autogamy, which serves as a reproductive insurance because outcross events are very rare.

INTRODUCTION

The long-term survival of a species may be dependent on its ability to adapt to changing circumstances. In particular, the evolution of self-fertilisation in angiosperms may be selected for when pollinators are scarce (Darwin 1876, Baker 1955, Jain 1976, Lloyd 1980, Holsinger 1996), even if inbreeding depression is strong (Schoen and Brown 1991, Lloyd 1992, Schoen *et al.* 1996). However the evolution of selfing as a reproductive assurance mechanism depends on selfed progeny having some fitness and that selfing does not occur at the expense of cross pollination (Lloyd 1979). Reproductive assurance may be promoted through autonomous autogamy (within flower selfing) or facilitated autogamy ((within flower selfing caused by insects (Lloyd and Schoen 1992)). Although vector-facilitated selfing is a common mechanism leading to mixed mating systems (Richards 1986), Lloyd (1992) predicts that facilitated selfing is never of great benefit to plants as it provides very little, if any reproductive assurance. One of Lloyds (1992) primary reasons for this is that facilitated selfing is only of value when pollinators are very scarce. But since facilitated selfing requires pollinators, it does not provide assurance when it is most needed (Lloyd 1992). However, this assumes that the conditions, which limit outcrossing pollinators, also limit the pollinators that facilitate selfing.

Reproductive assurance can be tested by emasculating flowers. Emasculated flowers should set fewer seeds than intact flowers if seed set is limited by pollen from other flowers (Schoen and Lloyd 1992). Reproductive assurance can only operate when pollen from other flowers (geitonogamous and xenogamous) limits seed set. Furthermore, the benefits of reproductive assurance will be negated if selfed seed (which are assumed to be inferior to outcrossed seed due to inbreeding depression) preclude outcrossed seed (Herlihy and Eckert 2002). This is commonly called "seed discounting," the absence of which is important for reproductive assurance to be effective (Herlihy and Eckert 2002).

Although reproductive assurance is thought to be a primary factor driving the evolution of selfing, there have been few experimental field studies on whether self-fertilisation actually does provide reproductive assurance, and of these, few examine seed discounting (Schoen and Lloyd 1992). The importance of reproductive insurance is controversial because the majority of experimental studies have shown that xenogamous and geitonogamous pollen is not limiting and thus self-fertilisation provides no reproductive assurance (Bernhardt 1976, Cruden and Lyon 1989, Leclerc-Potvin and Ritland 1994, Klips and Snow 1997, Eckert and Schaefer 1998). Only two studies (Motten 1982, Piper *et al.* 1986) indicate that xenogamous and geitonogamous pollen may be limiting and that self-fertilisation can potentially provide reproductive assurance. The most comprehensive study to date (Herlihy and Eckert 2002) is the only study to combine experimental measures of reproductive assurance, seed discounting, selfing rates and inbreeding depression. They show that *in Aquilegia canadensis*, autonomous selfing increases seed production. However this benefit is outweighed by the loss of high quality seed due to seed discounting and inbreeding depression. Their results challenge the view that reproductive assurance leads to the evolution of selfing.

The other general hypothesis for the evolution of self-fertilisation is the automatic selection hypothesis (Schoen *et al.* 1996). Under this hypothesis, genes promoting selfing spread more rapidly through a population than those promoting outcrossing, because selfers are potentially more efficient pollen donors than outcrossers (Fisher 1941). However, the spread of genes promoting selfing is opposed by inbreeding depression which only allows selfing to evolve if the relative fitness of progeny from selfing is greater than half that of outcrossed progeny (Fisher 1941). One of the assumptions that may frequently be violated by many automatic selection models (Fisher 1941, Nagylaki 1976, Wells 1979, Holsinger 1991) is that selfing does not provide reproductive assurance (Holsinger 1996).

In this study, I examine the pollination biology of both species comprising the family Roridulaceae, experimentally testing whether facilitated autogamy can provide

reproductive assurance and thus be a strong selective pressure for the evolution of self-compatibility and self-fertilisation. *Roridula* is a plant genus found in small, isolated populations in the fynbos biome, South Africa (Obermeyer 1970). The fynbos biome is characterised by frequent fires that are thought to maintain species richness and diversity (Cowling *et al.* 1992) and a wide variety of plant responses have evolved to cope with these disturbances (e.g. many Restionaceae resprout after fire (le Maitre and Midgley 1992). *Roridula* plants do not resprout after fire. Instead, seed germination is stimulated by fire and a single cohort of seedlings recruit after each fire event. Thus, reliable seed set (and reproductive assurance) between fires is crucial.

The family Roridulaceae consists of only one genus and two geographically separated species, *R. gorgonias* and *R. dentata* (Obermeyer 1970, Carlquist 1976). Plants are sticky and catch large numbers of insect prey. Recent work by Ellis and Midgley (1996) revealed that *R. gorgonias* has a mutualistic relationship with a hemipteran (*Pameridea roridulae*). The carnivorous hemipterans *Pameridea roridulae* and *Pameridea marlothi* live permanently and obligately on *R. gorgonias* and *R. dentata* respectively (Dolling and Palmer 1991). They roam the plants, apparently unaffected by the sticky secretions, consuming snared prey (Dolling and Palmer 1991). *Pameridea* defecates on *Roridulas'* leaves and nitrogen is then absorbed through the leaf cuticle (Ellis and Midgley 1996, Anderson and Midgley 2002).

Marloth (1903, 1925) observed that juvenile *Pameridea* apparently play a role in the pollination of *Roridula*. However Lloyd (1934) expressed doubts regarding Marloth's anecdotal accounts of *Roridula* pollination. Johnson (1992) and Vogel (1978) also noted that *Roridula dentata* flower and anther structure corresponds to a buzz pollination syndrome (See plate 2) and Vogel (1978) postulated that *Roridula* is adapted to pollination by modern Apidae. However the sticky traps of *R. dentata* are easily capable of catching large Apidae (Marloth 1903). In addition, the peduncles of *R. dentata* are very short and flowers are set amongst sticky traps making for a difficult approach by bees (Marloth 1925). Marloth (1925) postulated that *Pameridea* pollinators are *Roridula*'s solution to the sticky trap problem. However, the large, bright showy flowers of *Roridula* seem "unnecessary" for the purpose of attracting pollinators that are already on the plant in large numbers (Lloyd 1934). The main flowering time of *R. dentata* extends from late June to November and that of *R. gorgonias* is from late June to late August. These flowering times span a large proportion of the Cape winter (June, July, August) that is typically cold and pollinators are therefore scarce.

In this study, I use a combination of field observations and floral manipulations to elucidate the pollen vectors in the system. By excluding each possible pollinator in

Plate 2 A. *Roridula dentata* flower. **B**. *Roridula gorgonias* flower. **C,D**. Poricidal anthers of *Roridula dentata* suggesting buzz pollination.

turn, I determine the contributions of bees and hemipterans to seed and fruit set. Using allozyme electrophoresis, I calculate the outcrossing rates of seed in unmanipulated flowers from several populations and explain the resultant genetic structure in terms of the pollination and dispersal biology of the plants. Finally, I use both floral manipulations and allozyme electrophoresis to show the contribution of facilitated autogamy to seed set in *Roridula*. This is one of the few studies to examine self-fertilisation and reproductive assurance (see Herlihy and Eckert 2002).

METHODS

Pollination

Visits to flowers by potential pollinators (excluding *Pameridea*) were noted from an observation point where approximately 400-600 flowers could be seen. Observations took place on sunny days when insect pollinators are most active and when the *Roridula* flowers were open. A total of 69 hours of observation over 2.7 flowering seasons (May - November, 17 days) were made for *R. dentata* at four different populations (Pop8, 15, 18, 20). Fifty-three hours of observations over 2.7 flowering seasons (June-August, 14 days) were made for *R. gorgonias* at five different populations (Pop1, 2, 3, 6, 7). Visits were recorded if insects alighted on the petals of the flowers. Small visiting insects were captured, killed and immediately placed onto a slide with molten glycerol jelly. The insects were examined at a later date for pollen using a dissecting microscope. Pollen found on the insects was compared to a slide of *Roridula* pollen taken directly from the flowers. Larger visiting insects were also caught and later examined for pollen using a dissecting microscope.

Flowers from one *R. dentata* population (Pop8, see map in introduction) were haphazardly examined every meter for the presence of potential pollinators (n = 275) along four bisecting line transects. Flower visiting fauna (including *Pameridea*) was noted and if possible specimens were caught. Insects were preserved and examined for pollen grains as above. Flowers from a *R. gorgonias* population (Pop1, see map in introduction) were also examined haphazardly for fauna and insects were preserved using the same methods as above. Since there were only fifty plants in this population, more than one flower was sampled per plant (n = 100).

Floral manipulations were performed to examine seed set due to autogamy and hemipterans. Eight different floral manipulations were carried out as follows: 1) Flying insects were excluded from the flowers by bagging a rosette of leaves with an unopened bud on it (*R. gorgonias*: n = 36, *R. dentata*: n = 30). Bags were made from very light, transparent nylon pantyhose material, supported by a thin wire frame (floristry wire), which kept the bag away from the flower. The bag was closed on both ends using cable ties. Resident hemipterans on the rosette stayed alive in the bag

presumably by sucking plant sap. At harvesting time, bagged rosettes still contained live *Pameridea*. 2) Anthers were removed from 30 *R. dentata* flower buds on 30 plants to estimate the contribution of geitonogamy and xenogamy to fruit/seed set. If reproductive assurance is occuring in this system, we expect that emasculation will cause a significant drop decline in seed or fruit set. However, in this system pollen is the only reward offered by *Roridula*, and emasculation can potentially make flowers less attractive to pollinators. Thus it is impossible to determine if decreased fruit/seed set is due to a limitation of xenogamous/geitonoganous pollen or due to flowers being less attractive to pollinators. Consequently the emasculation of *Roridula* can lead to the rejection of the reproduction assurance hypothesis if emasculated flowers reach full seed/fruit set. However, a decline in seed/fruit set of emasculated flowers cannot be taken as clear support for the hypothesis. 3) To test the effect petals have on pollinator attraction, the petals of 30 *R. dentata* flowers were removed. 4) As a control for this, the petals of another 30 *R. dentata* flowers were removed and the flowers cross pollinated. Stigmas at various stages of floral maturity were placed in hydrogen peroxide to determine if they were pollen receptive (receptive stigmas bubble vigorously). It was determined that stigmas become receptive just prior to flower opening. Cross pollination was achieved by shaking the pollen from several flowers into a small container. Just prior to opening, flower buds were manually opened and pollen was transferred from the container onto the stigmas. The anthers of these flowers were removed, preventing self-fertilisation. 5) All pollinators were removed from the system by bagging a flower and ensuring that there were no hemipterans in the bag (*R. gorgonias*: n = 22, *R. dentata*: n = 50). One and a half months after the treatments, the seed capsules were collected, dissected and their seeds counted. At the time of harvesting, 6) natural seed set (control) was examined by walking along transects and picking a seed capsule every meter, one arms length away (*R. gorgonias*: n = 96, *R. dentata*: n = 226): Seeds in the capsules were counted as above. As only 50 plants existed at the *R. gorgonias* study site, more than one capsule was taken per plant (approximately 3000 capsules existed in the population at the time of sampling).

In addition to the above flower manipulations, two more treatments were completed in order to determine the plants' breeding system: 7) hand crossing flowers (*R. gorgonias*: n = 16, *R. dentata*: n = 30) and 8) self pollinating (*R. gorgonias*: n = 16, *R. dentata*: n = 30): Flowers were cross pollinated using the same methods as treatment four. After pollination, flowers were bagged ensuring that no hemipterans were in the bags and this was checked again at the time of harvesting. The methods for selfing were similar to those for cross pollination except pollen from the same plant was used (geitonogamy). Floral manipulations 1,5,6,7 and 8 were performed on *R. gorgonias* and treatments 1-8 were performed on *R. dentata*. Treatments 2-4 were not applied to *R. gorgonias* because population size was small and these extra treatments may have had detrimental effects on the seed set of the population. The

effects of the treatments were compared using Fishers Exact Test and a sequential Bonferroni was calculated for significance values to correct for type-I errors (Rice 1989). The data entered were fruit set (i.e. the numbers of flowers from each treatment which set seed versus the number which did not). Mean seed set of each treatment was also calculated by counting the number of seeds with embryos in each fruit capsule.

An additional treatment was attempted where *Pameridea* were excluded from flowers but flowers were still accessible to flying pollinators. A very sticky, non-toxic substance (Formex) used to exclude ants from fruit trees was applied to the peduncles of flowers. However, the hemipterans were able to traverse this barrier and so the treatment was abandoned. This may represent a weakness in the results, as seed set by flying pollinators alone could not be accurately determined by floral manipulation. In addition, total exclusion of *Pameridea* from plants may affect seed set due to the inhibition of the nutritional mutualism.

Allozyme electrophoresis

I collected seed material from 11 *R. dentata* localities (Pop8, Pop15, Pop16, Pop17, Pop12, Pop14, Pop22, Pop18, Pop19, Pop20, Pop21) and six *R. gorgonias* localities (Pop1, Pop7, Pop2, Pop3, Pop6, Pop4). For geographic positions of study sites, see map (in introduction).). If plant populations were smaller 20 individuals, then one capsule was collected from each plant in the population. In larger populations, between 20 and 24 capsules were collected. In large populations, capsules were haphazardly picked along two perpendicular, bisecting transects from within half a meter of the transect line. The distance between samples depended on the population size. Seeds were placed on ice and taken back to the laboratory where they were frozen at -80°C. Endosperm from *Roridula* seeds was dissected out and this material was homogenized using the vegetative extraction buffer I of Cheliak and Pitel (1984). They were homogenized with a glass rod attached to a variable-speed electric motor. Samples were used within four weeks of being collected and they were centrifuged for 5 min at 12 000 $r.min^{-1}$ prior to use. Filter paper wicks (Whatmans # 3) were dipped into the supernant of centrifuged samples and these were inserted into 13 % horizontal starch gels. The amount of sample tissue from each seed was very small and a maximum of two gels were run per sample. Thus I was unable to obtain a complete genotype of all loci for each individual. However care was taken not to use more than one seed per capsule for each locus investigated.

The enzymes MDH (E.C. 1.1.1.37), ADH (E.C. 1.1.1.1), GPI (E.C. 5.3.1.9) and DIA (E.C. 1.6.2.2) were resolved on a continuous Histidine-citrate buffer system, pH 6.0 (Stuber *et al.* 1977). Pep LGG (E.C. 3.4.-.-), IDH (E.C. 1.1.1.42), ME and PGM (E.C.

5.4.2.2) were resolved using a discontinuous Tris-citrate-borate-lithium hydroxide buffer: with a gel buffer pH 8.7 and an electrode buffer pH 8.0 (Ridgeway et al 1970). G6 (E.C. 1.1.1.49) and MPI (E.C. 5.3.1.8) were resolved using a continuous Tris-borate-EDTA buffer system, at pH 8.6 (Markert and Faulhaber 1965). The enzymes PGM (one locus), MPI and IDH (both loci could only be resolved clearly for *R. dentata* and not *R. gorgonias*.

Genetic markers are useful for determining geneflow between and within populations. The genetic differentiation among populations can be roughly measured using F_{st}, which is influenced by mutation, migration, drift and selection (Barrett and Kohn 1991). Values vary between zero (panmictic populations) and one (total isolation between populations). Since plant geneflow is a function of both seed dispersal and pollen movement, I cannot separate the effects of the two modes of geneflow. But if geneflow between populations is low ($F_{st} \sim 1$), it is possible to infer that both seed dispersal and pollen movement between populations is low or absent. However, these statistics must be used cautiously as very strong disruptive selection may also influence F_{st} (Bossart and Prowell 1998). The genetic structure among populations was tested by Fisher's exact test. F_{st} was estimated according to Weir and Cockeram (1984) using the computer program Genepop (Raymond and Rousset 1995). Inbreeding can also be estimated using the inbreeding coefficient (F_{is}). If $F_{is} = 0$, there is random mating within a population, whereas a value of one would indicate total inbreeding within the population. Departures from the null hypothesis (random mating) were measured after applying the sequential Bonferroni correction (Rice 1989). F_{is} and significance values were also calculated using the program Genepop (Raymond and Rousset 1995). In addition, the selfing rate (s) was approximated using the equation $F_{is} = s/(2-s)$, Barret and Kohn (1991). F_{st} and F_{is} estimates gene flow within and between populations that in turn gives us important information about the biology and movement of the pollinators that contribute to plant gene flow.

RESULTS

The only regular visitors observed on *R. dentata* flowers were the resident hemipterans *P. marlothi* and thrips. *Pameridea* juveniles were present inside 27.3 % of all flowers examined, at an average of 0.3 ± 0.5 per flower. Of all hemipterans found in *R. dentata* flowers, 17.3% were carrying *Roridula* pollen. Four percent of hemipterans in flowers were adults. Thrips were found in only a small percentage of flowers examined (4%). Simulid flies were observed landing on the flowers on two separate occasions. Both were caught and examined for pollen but none was found. In addition, 10 simulid flies caught in the near vicinity of the plants were also examined for pollen, however none was found. Pollen was found on one of the ten thrips collected on *R. dentata*. On two occasions, an unidentified, grey bee (Anthophoridae) was observed buzz pollinating flowers, visiting two flowers before

flying off on each occasion. In addition a *Xylocopa sp.* (Anthophoridae) was seen visiting flowers on one occasion (Marius Brand, pers. com.). None of the above bees was captured.

During the observations on *R. gorgonias*, five species of insect visited the flowers. The most frequent visitors were *Pameridea* which were present in 60% of all open flowers examined and on average each flower contained 1.6 ± 1.8 hemipterans at any time. Of these hemipterans, 68% were found to be carrying *Roridula* pollen. Of all the hemipterans found in flowers, only 0.5 % were adults even though adults comprise approximately 7.5% of the total hemipteran population (pers. obs.). Hemipteran juveniles were observed feeding on pollen in the anthers when the anthers dehisced and the pollen spilled out. Thrips were also occasionally found in flowers although in comparison to *Pameridea* they were rare, only occurring on 0.6 % of all flowers examined. Six visits were recorded from the small bee *Allodape punctata* (Anthophoridae). Two visits from the carpenter bee, *Xylocopa albifrons* (Anthophoridae) were recorded (both bees became briefly entangled in the leaves). A visit by a blister beetle *Ceroctis capensis* (Meloidae) was recorded on a single occasion. This species seems to be opportunistic and was found feeding on a wide variety of flowers. Fifty percent of the visiting *A. punctata* bees examined were carrying *Roridula* pollen after they had landed on a *Roridula* flower. Only one *Xylocopa* was captured and it was also carrying pollen after landing on *Roridula*. During the observational hours several *Xylocopa* were seen flying past the *Roridula* populations but few ever landed. The blister beetle was observed flying between flowers and consuming floral parts. It was covered in *Roridula* pollen and was most likely cross pollinating flowers although at the same time potentially damaging flowers visited.

Floral manipulations

Trends in fruit set and seed set follow each other closely (Fig. 1). I refer to fruit set in the results and discussion. The removal of all potential pollinators (excluding *Pameridea*) from *R. dentata* resulted in no significant reduction of the fruiting success of flowers (treatment 1 vs. 6, $\chi^2 = 2.74$, $P > 0.05$), thus seeds produced in this treatment are either the product of autogamy or the result of *Pameridea* pollinators. But the removal of all potential pollinators (autogamous seed production) including *Pameridea* resulted in a 68% reduction in fruiting success (treatment 5 vs. 6, $\chi^2 = 75.38$, $P < 0.001$). This is the amount of seed set attributable to pollinators, primarily *Pameridea* (Fig. 1). The removal of *R. dentata* petals also resulted in a significant drop in fruit set (treatment 3 vs. 4, $\chi^2 = 8.29$, $P < 0.05$) suggesting that petals play an attractive role to pollinators. Fruiting success in the petal control treatment was not significantly lower than in hand crossed treatments indicating that the removal of petals caused no damage to stigmas or pollen (treatment 4 vs. 7, $\chi^2 = 19.72$, $P >$

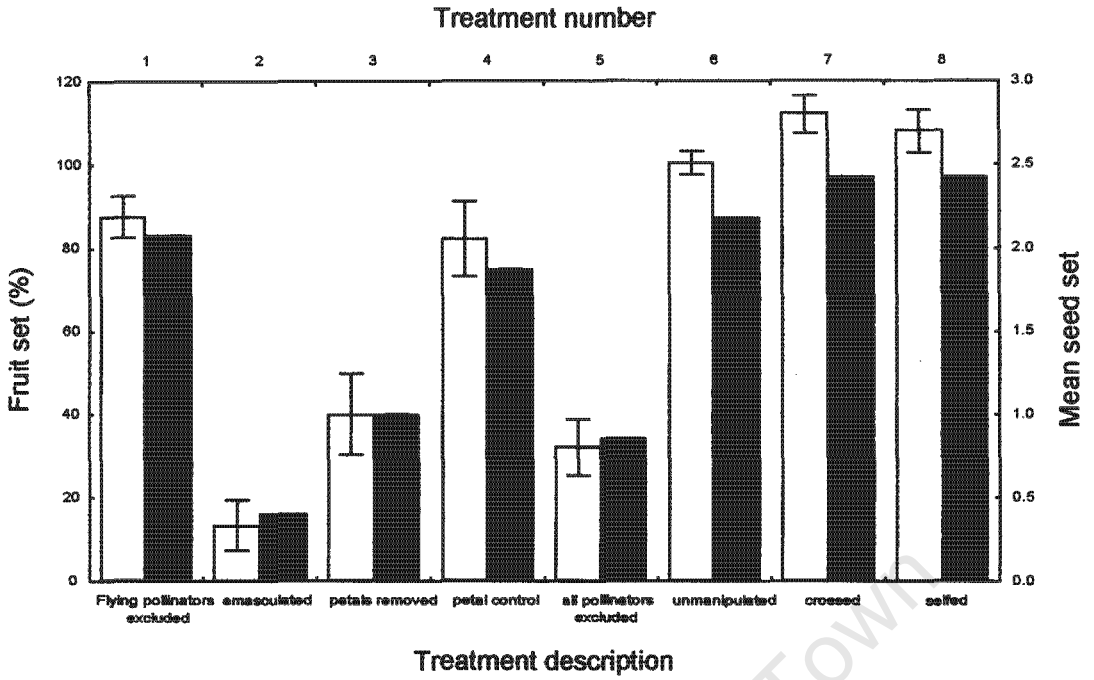

Figure1. Fruit set (shaded columns) and mean seed set (clear columns) after various floral manipulations in a population of *R. dentata*.

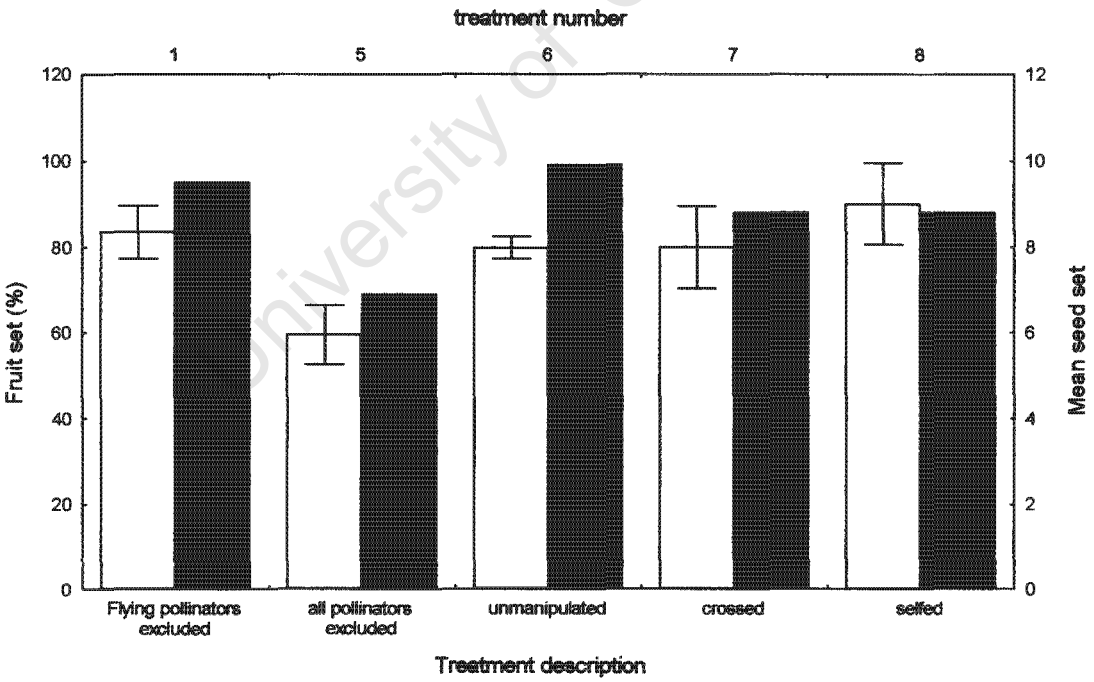

Figure 2. Fruit set (shaded columns) and mean seed set (clear columns) after various floral manipulations in a population of *R. gorgonias*.

0.05). Removal of petals produced the same seed set as when all pollinators were excluded (treatment 3 vs. 5, χ^2 = 0.29, P > 0.05), suggesting a visitation rate of close to zero. Emasculation of anthers ensured that no autogamy took place although pollinators still had access to flowers. This caused a large drop in fruit set (treatment 2 vs. 6, χ^2 = 91.66, P < 0.001) suggesting that most fertilisation events involved pollen from the same flower.

The exclusion of all pollinators (except *Pameridea*) from *R. gorgonias* resulted in no significant fruit set reduction (treatment 1 vs. 6, Fig. 2, χ^2 = 2.44, P > 0.05), almost all flowers in these treatments set fruit whereas the removal of all pollinators from the system resulted in significantly lower fruit set (treatment 5 vs. 6, χ^2 = 39.7, P < 0.001). This is a 25% reduction in seed set, and thus approximately 25% of seed set is attributable to pollinators, primarily *Pameridea*.

Crossed and selfed *R. dentata* flowers had equal success in fruiting (treatment 7 vs. 8, Fig. 1, χ^2 = 4.25, P > 0.05) and the mean seed set of these two treatments was also very similar (Fig. 1), suggesting that selfing is possible. Crossed and selfed *R. gorgonias* flowers were also equally successful at fruiting (treatment 7 vs. 8, Fig. 2, χ^2 = 100, P > 0.05). Differences in fruiting success of hand pollinated flowers versus unmanipulated flowers were non- significant (treatment 6 vs. 7, Fig. 2, χ^2 = 2.15, P > 0.05).

Allozyme electrophoresis

Pair wise F_{st} values of *R. dentata* (Table 1a) are generally large, even when the populations are close together. For example Pop15 and Pop16 are only 0.5 km apart but have an F_{st} of 0.4286 (P < 0.0001). Populations over 40 km apart have F_{st} values that tend towards one (P < 0.0001). Populations Pop17,Pop12 and Pop14 were all fixed for the same alleles and thus the significance values of paired F_{st} could not be calculated. Only one paired F_{st} below one was calculated where populations were not fixed for all loci. This relatively low pairwise F_{st} (0.0746) was nevertheless significant (P < 0.01) and was found in the northern region where populations are 13 km apart. Five populations (Pop17, Pop12, Pop14, Pop21 and Pop8) have fixed alleles for all loci examined and their F_{is} values could not be calculated. Populations where diallelic loci were scored all differed significantly from the null hypothesis of random mating (Table 2). Selfing rates in all of these populations were very high, ranging from 0.918 to one (Table 2).

Eight of the nine loci scored for *R. gorgonias* were monomorphic and fixed for the same allele through out all populations. Thus only one polymorphic locus was examined (MDH). Two alleles were found in MDH and populations Pop7, Pop6 and Pop1 are all fixed for allele "B." The population Pop4 is fixed for another allele, "C."

Populations Pop2 and Pop3 have combinations of both alleles and were the only populations where F_{is} and outcrossing rates could be calculated. Even though only one polymorphic locus was examined, strong structuring was evident between populations (Table 1b). However, the low numbers of polymorphic loci make it difficult to ascertain the true relationships between populations. Pair wise F_{st} values (Table 1b) vary from zero to one, but given that only one locus was polymorphic for two alleles, it is unlikely to find differences between all populations. Populations

Tables 1a and 1b. Pairwise F_{st} (below diagonal), pairwise geographic distance and significance above diagonal for *R. dentata*, separated into three regions (Table 1a), *R. gorgonias* (Table 1b). P > 0.05 = ns, 0.01 < P < 0.05 = *, 0.001 < P < 0.01 = **, 0.0001 < P < 0.001 = ***, P < 0.0001 = ****

1a

	South	Central					North				
	Pop8	Pop15	Pop16	Pop17	Pop12	Pop14	Pop22	Pop19	Pop18	Pop20	Pop21
Pop8	######	74****	74****	74****	59****	76****	138****	116****	116****	117****	134****
Pop15	0.9738	######	0.5****	0.9****	22****	4****	60****	52****	52****	52****	52****
Pop16	0.9298	0.4286	######	0.3****	23***	3*	60****	52****	52****	52****	52****
Pop17	1.0000	0.8916	0.3407	######	23 np	3 np	60****	52****	52****	52****	52****
Pop12	1.0000	0.8860	0.3258	0.0000	######	24 np	80****	63****	63****	63****	63****
Pop14	1.0000	0.8343	0.1958	0.0000	0.0000	######	58****	46****	46****	46****	46****
Pop22	0.9794	0.9417	0.8576	0.9724	0.9732	0.9551	######	13**	14****	11****	4****
Pop19	0.8770	0.8543	0.7481	0.8572	0.8626	0.7719	0.0746	######	2****	2****	7.5****
Pop18	0.9490	0.9169	0.8361	0.9319	0.9336	0.8989	0.8272	0.6347	######	2****	11****
Pop20	0.9636	0.9418	0.8758	0.9593	0.9610	0.9446	0.8875	0.6838	0.8102	######	7****
Pop21	1.0000	0.9621	0.8938	1.0000	1.0000	1.0000	0.9558	0.7673	0.8998	0.9005	######

1b

	Pop7	Pop6	Pop4	Pop3	Pop2	Pop1
Pop7	######	18 np	4.5****	5****	5****	2****
Pop6	0.0000	######	20.5****	21****	21****	19 np
Pop4	1.0000	1.0000	######	0.5 ns	0.8****	3.5****
Pop3	0.9333	0.9289	0.0000	######	0.3****	4****
Pop2	0.4108	0.3945	0.4585	0.3517	######	4****
Pop1	0.0000	0.0000	1.0000	0.9373	0.4260	#####

Table 2. F_{is} values and selfing rates for *R. dentata* populations (Pop15-20) and *R. gorgonias* populations (Pop2 and Pop3). * Denotes statistical difference from the null hypothesis of random mating (P < 0.001)

Population	F_{is}	selfing rate	#loci
Pop15	1.000*	1.000	1
Pop16	1.000*	1.000	1
Pop22	1.000*	1.000	1
Pop19	0.848*	0.918	3
Pop18	1.000*	1.000	1
Pop20	1.000*	1.000	1
Pop3	1.000*	1.000	1
Pop2	0.913*	0.955	1

Pop7, Pop6 and Pop1 were fixed for the same alleles and so pairings between these populations also had F_{st} values of zero. Significance values for pair wise F_{st} were not possible to calculate in these populations. High F_{st} values were found between some populations, which were very close. Pop2 and Pop3 are only 0.3 km apart and exhibit an F_{st} of 0.3517 ($P < 0.0001$). Populations Pop2 and Pop4 are also very close (0.8 km) and have a very high paired F_{st} of 0.4585 ($P < 0.0001$). Other populations, which are 3.5 to 4 km apart, have very high pair wise F_{st} values, which equalled one, indicating zero gene flow between these populations (Table 1b). F_{is} values for Pop2 and Pop3 were 1.000 and 0.913 respectively and both were highly significant ($P < 0.001$ in both populations) indicating non-random mating and inbreeding (Table 2). Selfing rates in both of these populations were also very high (Pop3 = 1.000, Pop2 = 0.913, Table 2).

DISCUSSION

Both species of *Roridula* are fully self-compatible and are able to self pollinate which may be important for "reseeders" like *Roridula*, especially if outcross events are rare. Bond (1994) recognised that plants with single pollinators often have "compensation" measures that ensure their persistence in the event of pollinator failure. Self compatibility and autogamy are likely to perform this role in *Roridula*. Although *Roridula* flowers are seemingly adapted to bee pollination, bees were rarely seen pollinating flowers, even though natural fruit set was very high. The exclusion of only flying pollinators from flowers (treatment 1) did not decrease fruit set. Instead, fruit set was maintained in this treatment at values close to natural fruit set because hemipterans were still able to forage in flowers and they facilitated pollination. Since the hemipterans were constrained by nylon bags to a single flower, fruit set was autogamous. In contrast, the removal of all pollinators including *Pameridea* resulted in a very large drop in the fruiting success of both *Roridula* species. This suggests that self pollination in the absence of *Pameridea* is not very efficient and also that *Pameridea* is an important pollinator and can account for 68% of fruit set in *R. dentata* and 25% in *R. gorgonias*. The majority of hemipterans found in flowers were juveniles that were small (< 3mm), wingless and not very motile (per. obs.). Due to their wingless state, juvenile *Pameridea* are unlikely to move pollen between plants and are therefore only likely to contribute geitonogamous pollen or pollen from the same flower.

Using allozymes, I found that where F_{is} and selfing rates could be calculated, very high degrees of selfing were always suggested in populations of both *Roridula* species. Thus *Roridula* is predominantly a selfer and outcrossing events are extremely rare in all populations tested. This is consistent with field observations and floral manipulations that show that the dominant pollinators are sessile hemipterans. Bees are likely to have a large outcross component to their pollination but due to their

scarcity as pollinators, outcrossed seeds were very rare. F_{st} values are also generally very high (even between some close populations) suggesting that both pollinator movement and seed dispersal occur on a very small scale in both species. A single exception was found between the *R. dentata* populations Pop19 and Pop22 where F_{st} was below 0.1 although the populations were still significantly different. This low F_{st} value could be the result of either pollen or seed movement and may indicate recent ancestry between the populations. Some of the bees seen visiting *Roridula* (especially *Xylocopa*) are large, powerful fliers, capable of travelling long distances (Whitehead, pers.com.). Despite this, their contribution to geneflow and pollination must therefore be very low and infrequent in most of the populations examined.

In this system, outcrossed pollination events cannot account for the high fruit seed set observed in *Roridula*. Even with the addition of autogamous selfing, fruit set is well below the observed natural fruit set. Levels of natural fruit set can only be attained in the populations analysed by a combination of autonomous selfing and pollination by juvenile hemipterans (almost entirely selfed or geitonogamous). Thus autonomous selfing and facilitated selfing are likely to play important reproductive insurance roles in *Roridula*.

One of the perceived obstacles to facilitated selfing offering reproductive assurance, is that it is still reliant on pollinators at times when pollinators are supposedly scarce. However, in this system, potential pollinators may be very common and reliable except that they produce selfed progeny. Other, more motile pollinators may be more scarce and affected by environmental conditions. A similar system was studied by Baker and Cruden (1991) where thrips reliably pollinated *Potentilla* in the absence of more motile pollinators. Zamora (1995) found a similar situation where thrips were important pollinators of *Pinguicula* in shady areas but not sunny areas when larger pollinators were more common. Baker and Cruden (1991) hypothesised that pollination by non motile pollinators such as thrips may be an important and widespread phenomenon. However, Lloyd (1992) refers to these small pollinators as squatters because they spend large amounts of time in flowers. He also suggests that squatters are generally not selected for because they may have detrimental effects on plants and their movements are often unpredictable. However, in the *Roridula* system, large numbers of hemipterans are found on every plant and are extremely prevalent in all life stages, all year round (pers. obs.). This is important for both *Roridula* species because much of their flowering seasons stretch through winter when other pollinators are dormant or inactive (e.g. Hepburn and Jacot-Guillarmod 1991). *Pameridea* are also species specific as they only occur on *Roridula*. As a result, *Roridula* loses no pollen to the stigmas of other plant species and pollen from other plants does not clog *Roridula*'s stigmas. Another drawback of facilitated selfing is that it may often lead to seed discounting where self pollination of

ovules may pre-empt ovules that would otherwise have been outcrossed (Lloyd 1992). However, this disadvantage is minimized when cross pollination events are extremely rare or absent. In addition, *Roridula* has weakly delayed self pollination where the stigma becomes receptive approximately one day before the anthers (pers. obs.). This allows geitonogamous and xenogamous pollen a temporal advantage over autogamous pollen.

One of Lloyd's (1934) arguments against hemipterans being pollinators of *Roridula* was the presence of showy petals. The need for advertising is another factor thought to limit the advantages of facilitated selfing because it is more expensive than autonomous modes of selfing (Lloyd 1992). This study shows that petals still have a vital function because their removal causes a large drop in fruit set. Petals still attract rare bee pollinators, which may be important for rare outcross events. The large reduction in seed set caused by removing petals suggests that petals are also attractive to resident *Pameridea*. It was noticed that the flowers of *R. gorgonias* close at night whereas those of *R. dentata* do not (pers. obs.). Large numbers of juvenile hemipterans were observed sheltering in *R. gorgonias* flowers at night (pers. obs.) and so flowers may also act as a refuge for *Pameridea*. *R. dentata* occurs in a much drier and hotter part of the country than *R. gorgonias* and its flowers are pendulous whereas the flowers of *R. gorgonias* face the sun (pers. obs.). Flowers of *R. dentata* can be used by hemipterans in a thermoregulatory way and the umbrella of petals may be used by hemipterans as shade. These results suggest that increased seed production alone (i.e. without lower advertising costs) is enough of a benefit for the evolution of selfing.

Emasculating flowers caused a large drop in the fruit set of *R. dentata* that is consistent with the reproductive assurance hypothesis. However pollen is the only reward offered by *Roridula* and emasculations may make flowers less attractive to pollinators. It is impossible to determine how much of this decrease was due to the loss of selfing ability and how much was due to flowers becoming less attractive to pollinators. In this system emasculation only has the ability to disprove the reproductive assurance hypothesis (if emasculated flowers reach full seed set) but not prove it. These emasculation results do not disprove the hypothesis.

The evolution of self-compatibility in rare plants is thought to be favoured by certain aspects of their population biology (Karron 1991). In small populations, mates may be scarce or closely related. In such populations, outcrossing within a population should have very little advantage over selfing. In addition, since relatedness may be high in small populations, matings between unrelated individuals would be uncommon. Hence obligate outcrossing may be selected against. Thus, small population size may favour the evolution of self compatibility, but not necessarily

autogamy. Many *Roridula* populations number between five and 50 individuals, possibly necessitating self compatibility.

Self compatibility is an important precursor for the evolution of autogamy. Autogamy may be stimulated by shortage or competition for pollinators (e.g. Wyatt 1988) or as a mechanism to speed up the development of seeds (e.g. Pedersen and Ehlers 2000). However, the effects of inbreeding depression are sometimes thought to constrain the evolution of autogamy and it has been demonstrated that mating with close relatives in animal and plant populations often causes inbreeding depression (Ralls *et al.* 1979; and van Treuren *et al.* 1993), which can lead to extinction (Saccheri *et al.* 1998, Keller and Waller 2002). A commonly assumed genetic basis for inbreeding depression is the presence of deleterious recessive alleles that are frequently expressed with the onset of selfing (Charlesworth and Charlesworth 1987, Wright 1977). Recessive alleles may be purged through natural selection and high levels of prolonged inbreeding (Barrett and Charlesworth 1991; Barrett and Kohn 1991). Thus, in some plants, which habitually self fertilize, the effects of inbreeding depression are minimal or non-existent (Proctor *et al.* 1996). However, such plants may have survived a period of high inbreeding depression as a result of deleterious alleles being expressed. If this is the case, then it is likely that *Roridula* plants have a history of purging: *Roridula* plants are probably closely related as populations are often isolated with fewer than 100 individuals and geneflow between populations is very low. Thus *Roridula* needs to be tolerant of inbreeding and self compatibility is a logical consequence of mating with closely related individuals. *Roridula gorgonias* is also habitat restricted and the seeps in which it occurs are often small and unable to harbour larger populations. Therefore the ability to mate with closely related individuals might date back as far as the plants association with moist nutrient poor habitats which are in turn associated with carnivory. Given a likely history of inbreeding, inbreeding depression may not affect *Roridula* very seriously. Although Proctor *et al.* (1996) suggest that plants with partially or normally inbreeding populations will suffer little inbreeding depression due to purging, theoretical results and data of Charlesworth and Charlesworth (1987) suggest that even highly inbred populations may suffer from inbreeding depression. Recently models have been developed which indicate that genetic load (due to mildly deleterious alleles) increases as population size decreases because the efficiency of selection is decreased by the effects of genetic drift (Bataillon and Kirkpatrick, 2000) and "purging" may not be very effective.

Although the long-term effects of inbreeding are difficult to determine in this study, I nevertheless show that selfing, in particular facilitated selfing is necessary for flowers to reach their full seed set. It is most likely that facilitated selfing in this system acts as a reproductive insurance that guarantees seed production in the likely event of pollination failure by bees. These are among the first data to show that facilitated

selfing may be an important reproductive assurance mechanism in some systems, although this mode of reproductive assurance may be rare in other systems (Lloyd 1992). However, I believe future work needs to show that selfed seeds have some fitness (i.e. they can germinate and reproduce). Nor does this study show the effect of seed discounting. However outcrossing seems to be so rare that seed discounting is uncommon. Furthermore, stigmas ripening before anthers may further reduce seed discounting. Comparative data on the fitness out outcrossed versus selfed progeny would also be important in determining whether facilitated selfing is advantageous to *Roridula*. This study also shows that the species-specific mutualism between *Pameridea* and *Roridula* may extend beyond simple nutritional benefits. This is one of the few examples of a mutualist pollinator that pollinates a single species of plant.

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2 Digestive mutualism, an alternate pathway in plant carnivory

ABSTRACT

The carnivorous status of *Roridula* is that of a near-carnivore as it does not fully satisfy the conditions for carnivory. *Roridula* has a mutualism with hemipterans, which facilitate prey digestion, although it is not known how much nitrogen *Roridula* derives from this association. I examine nutrition in *R. gorgonias*, using natural abundance levels of ^{15}N. *Roridula gorgonias* derives more nitrogen from insect sources (about 73 %) than most species of *Drosera*. Considering the effectiveness and specificity of the mutualism, *Pameridea* may be regarded as a functional "digestive organ." If *Pameridea* can be viewed as an unusual kind of digestive organ, then *Roridula* should be judged carnivorous. I challenge and modify the existing definition of plant carnivory.

INTRODUCTION

Carnivorous plants feature highly derived growth forms and they have frequently been used as model systems in the study of macromorphological evolution in angiosperms (e.g. Albert *et al.* 1992). Carnivorous plants have also been used extensively as models for ecological research (see Ellison *et al.*, in press). Other evolutionary studies have developed models explaining when and why plants should evolve carnivory (Givnish *et al.* 1984, Benzing 1987) and have even focused on the strange conflict between pollination and prey capture (e.g. Zamora 1999, Ellison and Gotelli 2001, Anderson and Midgley 2001). However, all these studies require a precise definition of what constitutes a carnivorous plant. Until now, most biologists have used the definition proposed by Givnish *et al.* (1984). They view the carnivorous syndrome as a continuum, with "unequivocal carnivores" at the one end having several adaptations to the carnivorous syndrome. At the other end of the continuum are "near-carnivorous" and non-carnivorous plants that show very few or no adaptations to carnivory.

The existing definition of carnivory

According to Givnish *et al.* (1984), carnivorous plants must fulfil two requirements: 1) they must be able to absorb nutrients from dead animals next to their surfaces. 2) They must possess morphological, physiological, or behavioural features whose primary effect is attraction, capture or digestion of prey. Furthermore "plants capable of absorbing nutrients from dead animals, but which lack active means of

prey attraction and prey digestion, and posses neither motile traps nor passive structures like one-way passages whose primary result is immobilisation of animals near plant surfaces must be considered saprophytes and not carnivorous plants" (Givnish *et al.* 1984). Although this definition includes most widely recognised carnivorous plants, it excludes the family Roridulaceae (See Juniper *et al.* 1989), which seems to derive a large proportion of its nitrogen from insects (Anderson and Midgley 2002). The definition may also exclude some Bromeliads, which are borderline carnivores and also relegates the pitcher plant *Heliamphora* to marginal carnivory (Juniper *et al.* 1989).

Roridula - *a carnivorous plant excluded by definition*

I use the family Roridulaceae to challenge and add to the existing definition of carnivory. The Roridulaceae have highly developed, sticky traps (Plate 3) that capture large numbers of insects (Ellis and Midgley 1996), fulfilling the second of Givnish *et al's.* (1984) requirements for carnivory. The leaves are long and slender, up to six centimetres in length and each is densely covered by stalked glands or tentacles ranging in length from minute to about 3.5 mm (Bruce 1907). Similar to *Drosera*, these hairs are glandular and each produces a sticky droplet to which the prey adheres. Unlike *Drosera* the, droplets are not protein based. Instead, they are a resinous compound (Marloth 1925), which is far more adhesive than the droplets produced by *Drosera*. However, the droplets produced by *Roridula* have no digestive enzymes to digest prey (Ellis and Midgley 1996) and hence there are no obvious morphological adaptations for prey digestion (point 1). Despite the fact that it captures large amounts of prey (Plate 3, Marloth 1903) it does not have any obvious adaptations for prey attraction. As a result, biologists have postulated that *Roridula* does not gain significant amounts of nutrients from trapped insects (Marloth 1925, Lloyd 1934). However, recent work suggests that obligately associated hemipterans (*Pamerídea*) have a digestive mutualism with *Roridula* (Plate 3). Two species of *Pamerídea* (Miridae) are known and each has an obligate species-specific association with a single *Roridula* species of which there are also two (Dolling and Palmer 1991). Unlike other insects, *Pamerídea* were able to walk unhindered over the sticky traps, consume prey caught by the plants and defecate on the plant leaves (Plate 3). *Roridula* then absorbs the faecal nitrogen through the thin plant cuticle. Anderson and Midgley (2002) have calculated that approximately 70 % of the nitrogen in *Roridula dentata* can be absorbed in this way. They suggest that the relationship between *Pamerídea* and *Roridula* may be obligately mutualistic for both partners, a very rare natural phenomenon (Thompson 1994).

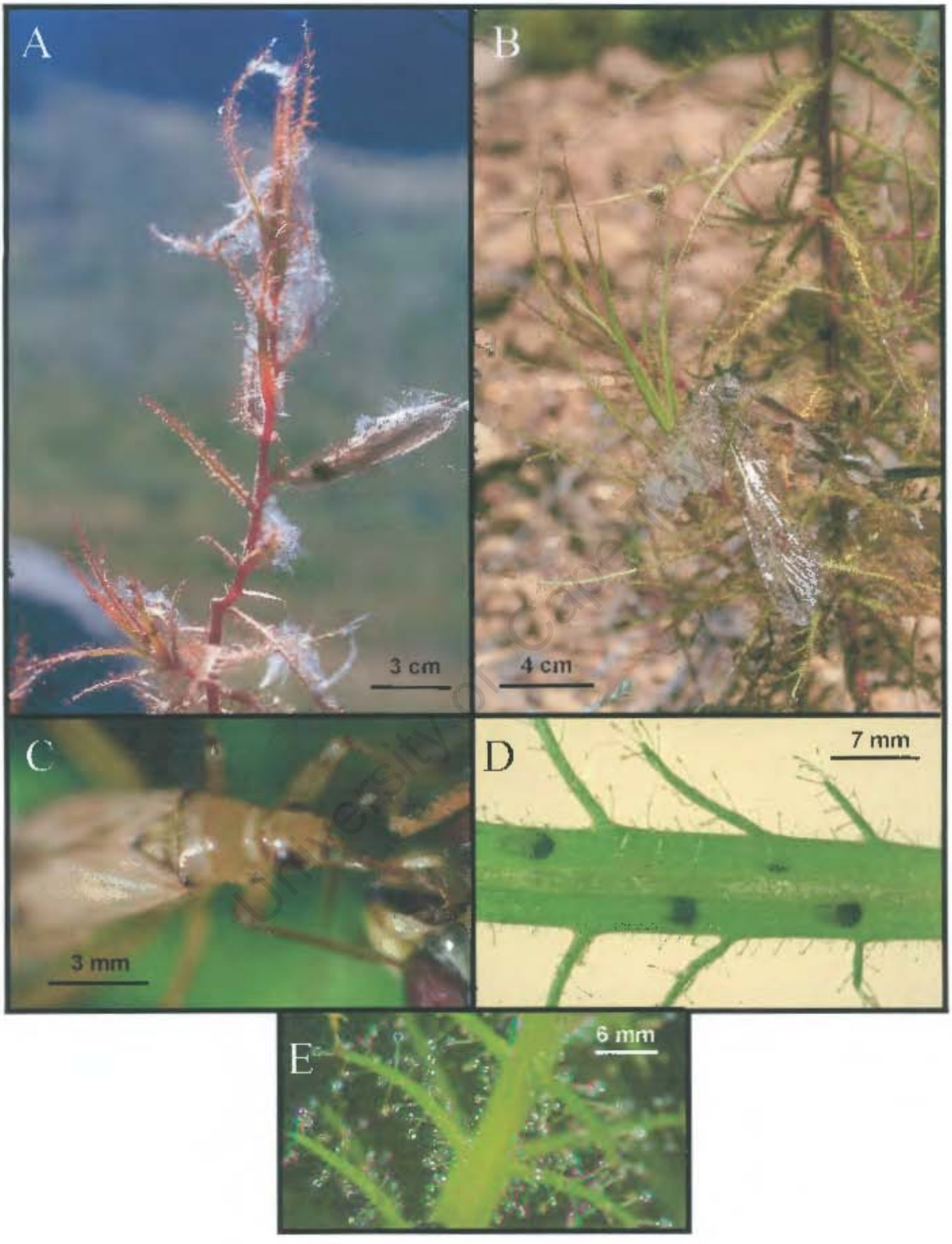
METHODS

Calculating the degree of dependence on insects

By using natural abundance levels of ^{15}N (see Anderson and Midgley 2002), which were modified from Schulze *et al.* (1991), I aim to quantify approximately how much nitrogen *Roridula gorgonias* derives from insects. The only work done previously on *Roridula gorgonias* nutrition are two studies (Ellis and Midgley 1996; Midgley and Stock 1998), neither of which made an attempt to quantify carnivory. However, Anderson and Midgley (2002) investigated nutrition in the other species of *Roridula* (*R. dentata*) using natural abundance levels of ^{15}N. They found that in addition to *Pameridea*, spiders also inhabit the plants. The densities of spiders on *R. dentata* control the *Pameridea* densities and therefore the amount of nitrogen that plants acquire from insects. They found that depending on spider and hemipteran densities, between 40-70 % of plant nitrogen could come from hemipteran faeces. I aim to approximate how much nitrogen *Roridula gorgonias* derives from having hemipteran mutualists (i.e. how important is the mutualism to *Roridula gorgonias*), especially since this species does not have spiders to decrease the efficiency of "indirect-carnivory".

Roridula gorgonias was studied at Vogelgat Nature Reserve (34°23' S, 19°19' E). The habitat contains a natural seep with permanently waterlogged, peaty, quartic sands and it is one of the largest known populations of *R. gorgonias.* To compare natural abundance levels of $\delta^{15}N$, five *Roridula gorgonias* leaf samples were collected, as well as reference plants at Vogelgat Nature reserve. Young leaves were bagged in a transparent nylon material while still in bud to exclude prey items and *Pameridea.* They were harvested approximately one week after bagging. Leaf samples were used instead of whole plants as *Roridula* is a Red data book species. Two non-carnivorous, VAM (based on Allsopp and Stock 1993) reference species, *Villarsia capensis* (Gentianaceae), and *Ursinia quinquepartita* (Asteraceae) and two species of *Drosera* were chosen from the study site. Leaf samples (new leaves) were collected from each of these (n = 5 plants) after being bagged in the same way. A third non-carnivorous plant, *Erica fastigiata* (Ericaceae) which has an ericoid Mycorrhizal system, was also included in the analysis for comparative reasons. Rooting depth is constrained by the study site having a very shallow soil layer and a high water table (see Midgley and Stock 1998). The poor root system of *Roridula* is comparable to the herbaceous reference plants *Ursinia* and *Villarsia.* The two *Drosera* species included *D. trinervia* with a rosette growth form and a very shallow rooting depth, and *D. hilaris*, which has an upright growth form and a rooting depth more comparable to the young *Roridula* plants in this study. Living prey was taken

Plate 3. The sticky leaves of *Roridula dentata* are capable of capturing very large prey items. Feathers indicate a struggle between plant and bird (**A**) and frequently large insects such as this neuropteran are found in amongst the leaves (**B**). **C**. *Pameridea marlothi* sucks the juices from a captured fly. **D**. *Pameridea* faeces on the leaf of *Roridula dentata*. **E**. Sticky traps on *Roridula dentata*.



from the leaves of *R. gorgonias* in order to obtain a signature for this possible nitrogen source. Prey consisted of eight Diptera, five Hymenoptera, three Coleoptera, and three Hemiptera.

I used the mass spectrometer methods of Anderson and Midgey (2002) to indirectly determine the $\delta^{15}N$ values of hemipteran faeces and the percentage of plant nitrogen obtained from those faeces. Anderson and Midgley (2002) show that indirect measurements of faecal $\delta^{15}N$ are reasonable approximations. In addition, I also calculated the percentage of nitrogen obtained by *Drosera* from insects using the methods of Schulze *et al.* (1991).

RESULTS

Roridula gorgonias had the second highest $\delta^{15}N$ value of all plants measured (Fig. 1) and is significantly different to the VAM plants in the study (χ^2 =8.57, P = 0.014, Kruskal-Wallis). The average $\delta^{15}N$ value for *Pameridea* is $\delta^{15}N$ = 9.12 ‰ and $\delta^{15}N$ = 5.04 ‰ for prey items. If insects are used as *R. gorgonias'* alternative food source (i.e. methods of Schulze *et al.* 1991), then the percentage nitrogen derived from insects is approximately 43.8 %. However if *Pameridea* faeces are used (i.e. methods of Anderson and Midgley 2002), then approximately 73.2 % of *Roridula* nitrogen is gained from hemipteran faeces.

Erica had the lowest $\delta^{15}N$ values recorded in the study (Fig 1), possibly reflecting that this species has a different mycorrhizal association to the other two VAM plants. The two VAM plants, *U. quinquepartita* and *V. capensis* were $\delta^{15}N$ = -4.95 ‰ and $\delta^{15}N$ = -5.30 ‰ respectively. The average of the two VAM plants was used as the reference plant value ($\delta^{15}N_{REF}$ = -5.125 ‰). The highest $\delta^{15}N$ value measured from plants at Vogelgat was that of the upright *Drosera hilaris* ($\delta^{15}N$ = 1.83 ‰, Fig. 1). Using the methods employed by Schulze *et al.* (1991), (modified from Peterson and Fry 1987) to determine carnivory derived from insects, I estimate that approximately 68% of nitrogen in *D. hilaris* is from an insect source.

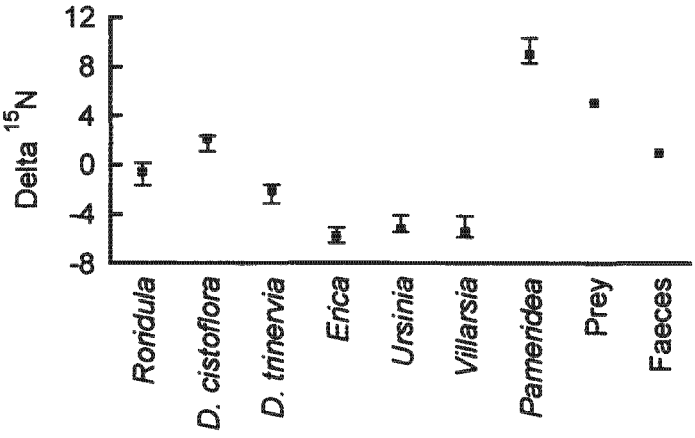


Figure 1. Delta ^{15}N values (medians ± maximums and minimums) for *Roridula gorgonias*, co-occurring carnivorous and non-carnivorous reference plants, *Pameridea*, prey and faecal values.

DISCUSSION

Carnivorous plants don't need conventional digestive organs

Although δ^{15}N values for *Roridula* are lower than those for *D. hilaris*, these plants have different nitrogen sources (faeces versus insects). By using faeces (instead of insects) to calculate carnivory in *R. gorgonias* I calculated that *Pameridea* faeces may be the source of over 70 % of plant nitrogen which is comparable to and exceeds the values of insect nitrogen in most other carnivorous plants (Dixon *et al.* 1980, Schulze *et al.* 1991). The most insect dependent carnivorous plants known are *Dionaea* and *Darlingtonia* with insect nitrogen levels of 75 % and 76 % respectively (Schulze *et al.* 1997, Schulze *et al.* 2001). *Roridula gorgonias* is almost equally dependent on insect prey! The levels of carnivory in *R. gorgonias* are also comparable to those of *R. dentata* (70 %) when there were high hemipteran densities and low spider densities (Anderson and Midgley 2002). Non-carnivorous plants all had extremely low δ^{15}N values. These values were more negative than reference plants in the studies by Schulze *et al.* (1991), possibly reflecting the mycorrhizal affiliation of plants (e.g. Hobbie *et al.* 2000). Alternatively, low δ^{15}N values could be due to the waterlogged nature of the soils, which are nutrient poor, shallow and possibly have slow rates of mineralization. The upright *D. hilaris* was the only plant with a positive δ^{15}N value and was consistent with the observations of Schulze *et al.* (1991) who found that upright growth forms of *Drosera* are more "carnivorous" than rosette forms. In this study, the rosette form of *Drosera* had a δ^{15}N value of δ^{15}N = -2.25‰ (significantly lower than *R. gorgonias* $P < 0.05$). The percentage of nitrogen derived from insects by *D. hilaris* was as high as some of the very carnivorous species examined by Schulze *et al.* (1991).

The evolution and persistence of digestive mutualism

The evolution and persistence of obligate, species-specific mutualisms are perhaps dependent on the net outcomes of a relationship being predictably positive for both partners through space and time (Thompson 1994). In this system, predictability may depend on hemipterans always being present on *Roridula* and not cheating. I have found hemipterans to be present on every *Roridula* population examined (except those that have been recently burned), although densities may vary significantly (Anderson and Midgley 2002). However, even at the lowest recorded hemipteran densities, Anderson and Midgley (2002) show that *Roridula* plants gain substantial amounts of nitrogen from hemipteran faeces. I also expect that cheating (i.e. not defecating on the plant leaves) in this system is negligible, as there does not seem to be an obvious cost to this behaviour. I expect that other important, obligate, species-specific digestive mutualisms should also have fairly stable outcomes in space and time.

Modifying the existing definition of carnivory

Despite the very sticky traps and large numbers of prey caught, the carnivorous status of *Roridula* is still controversial using the existing definition of carnivory. Ellis and Midgley (1996) showed that it is able to absorb nutrients from insects through its surfaces. However absorption in *Roridula* is an indirect process, even though it is extremely effective. In the strictest sense, *Roridula* is not truly carnivorous because prey digestion is not a direct process. However, *Roridula* does have several morphological features primarily for prey capture. But this adaptive feature alone is not enough to classify *Roridula* as being carnivorous. *Roridula* possesses many similarities to some *Sarracenia* species, which also have digestive associations (Juniper *et al.* 1989). Although the arthropods associated with *Sarracenia* are not as host-specific as *Pameridea* (See Juniper *et al.* 1989, Dolling and Palmer 1991). *Sarracenia* does not produce digestive enzymes and relies on bacteria and perhaps macro-fauna for prey digestion. Nevertheless, it is still regarded as a carnivorous plant because it has other adaptations to carnivory such as prey attraction mechanisms (Juniper *et al.* 1989). Based on the sticky trapping mechanism and the degree of nitrogen absorption from prey, I suggest that *Roridula* should also be judged carnivorous. But in order to do so, it may be necessary to broaden the definition of a digestive organ. By recognising species-specific digestive mutualists (such as *Pameridea*) as functional digestive organs or adaptations to carnivory, *Roridula* may fall within the guidelines for carnivory proposed by Givnish *et al.* (1984) and Givnish (1989).

By allowing more leeway on what constitutes a digestive organ or an adaptation to carnivory, *Sarracenia* may also be shown to have functional digestive organs formed by species-specific mutualists. Plants that incidentally absorb animal nutrients (e.g. bird droppings on a leaf or from a decaying animal) cannot be considered carnivorous. Therefore, I suggest that digestive mutualisms should only be considered equable to digestive organs if they are obligate and host-specific. This will affect very few plants because obligate, host-specific mutualisms are extremely rare (Thompson 1994). More specifically, the marginal carnivores such as *Heliamphora*, some Bromeliads (e.g. *Catopsis* and *Brocchina*) and other clearly carnivorous genera without digestive enzymes such as *Sarracenia* and *Darlingtonia* may be shown to have species-specific digestive mutualisms with invertebrate taxa. Hemipterans from the family Miridae have also frequently been closely associated with other carnivorous plants including *Drosera* (China 1953), *Byblis* (China and Carvalho 1951) and *Pinguicula* (Zamora 1995). Their role is always assumed to be commensal or klepto-parasitic, however, it seems that their roles could perhaps be re-evaluated.

Similar to *Roridula*, these mutualisms may prove to be highly evolved adaptations to carnivory. Further studies are needed to answer whether the interaction between Roridula and its Mirid partner is a rare or unique plant-animal interaction or whether this is an example of a common but previously unexplored pathway for plants to acquire nutrients from animals.

It is well known that adaptations to carnivory such as the production of traps or digestive glands are costly and lower the efficiency of botanical carnivory (e.g. Pate 1986, Mendez and Karlsson 1999). Alternative methods of prey digestion (e.g. digestive mutualisms) may represent specialized adaptations to the carnivorous syndrome because they reduce the costs of having to produce digestive structures and enzymes. Such reductions in costs may make carnivory more efficient and allow some carnivorous plants to occupy more marginal habitats.

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3 Cuticular adaptations to foliar absorption in *Roridula*

ABSTRACT

Roridula **plants capture insects but have no digestive enzymes. It is hypothesised that *Roridula* leaves absorb nitrogen from the faeces of obligately associated, carnivorous hemipterans. But rapid diffusion across the leaf surfaces of most plant leaves is prevented by the presence of an impermeable cuticle. However, in carnivorous plants, cuticular gaps or pores in digestive/absorptive cells allow rapid diffusion across the leaf surface. Using the dye neutral red, I found that aqueous compounds diffuse rapidly across the cuticle of *Roridula's* leaves but not across the cuticles of co-occurring, non-carnivorous plant leaves. Furthermore, immature *Roridula* leaves were unable to absorb neutral red whereas mature leaves were. Using TEM, cuticular gaps and pores similar to those in other carnivorous plants were found in the epidermal cells of mature *Roridula* leaves. The leaf cuticle of *Roridula* is also very thin and cell wall elements project close to the leaf surface, possibly enhancing absorption. Similar cuticular gaps are frequently found in absorptive cells of other carnivorous plants.**

INTRODUCTION

Roridula catches large numbers of insects using its very sticky traps that are superficially similar to those found in *Drosera*. The plants are able to assimilate insect nitrogen (Ellis and Midgley 1996) despite the fact that they have no digestive enzymes (Marloth 1910, Lloyd 1934, Ellis and Midgley 1996). Ellis and Midgley (1996) suggest that indirect carnivory after leaf fall or by fungi/bacteria is unlikely. The primary reason for this is that a carnivorous hemipteran lives obligately in very close association with *Roridula* (Dolling and Palmer 1991) and consumes prey within hours of capture, leaving behind only the exoskeleton. They suggest that *Roridula* absorbs nitrogen from the liquid hemipteran faeces, directly through the cuticle. In support of this, nitrogen from labelled flies was absorbed extremely rapidly (within 72 hours), making leaf fall and fungal contributions unlikely. In addition, labelled nitrogen was only incorporated into plant tissue when hemipterans were present, but not when hemipterans were excluded from plants. However, it remains to be determined whether *Roridula* has cuticular adaptations to make such rapid nutrient absorption possible.

The cuticle is the aerial layer covering all terrestrial, higher plants (Viougeas at al 1995) and it is characterised by hydrophobic properties that have a protective function (Schonherr 1982). A layer of epicuticular wax covers the leaves of all terrestrial plants and this layer can be amorphous to crystalline (Baker 1982), dense or diffuse. Below this is the cuticle, which can be divided into two layers: The upper layer is the cuticularized layer, which consists entirely of lipid material, with no cellulose or cell wall material (Martin and Juniper 1970). Below this is a cutinized layer (Martin and Juniper 1970), which may contain permeable cell wall elements (Lyshede 1978, Juniper *et al.* 1989). The cell wall, which is composed of polysaccharides and small amounts of protein, lies below the last cuticle layer. The presence of the cuticle effectively forms a transport barrier into and out of the leaf for hydrophilic substances (Price 1982) and this may pose a problem for the glandular cells (e.g. in carnivorous plants), which specialize in absorption or digestion. The conflicting roles of epidermal cells are highlighted in carnivorous plants whose digestive and absorptive cells must be highly permeable but still provide a barrier against water loss (Schonherr and Schmidt 1979) and pathogens (Tulloch 1976). The cuticle of digestive/absorptive structures is often very thin and this is thought to aid rapid diffusion across this layer (Helsop-Harrison 1976, Robins 1976). However, there are situations in which thicker cuticles are more permeable than thinner ones, which suggest that other factors may also influence transport across the cuticle (Norris 1974, Price 1982). Evidence suggests that the cuticle is not always a homogeneous lipid layer. For example carbohydrate fibres may extend into the cuticle from the cell wall and middle lamellae (Norris and Bukovac 1968) and these fibres may provide hydrophilic pathways, which extend from the cell walls to the proximity of the cuticle surface (Hoch 1979). Cuticular discontinuities in carnivorous plants have also been identified in the form of gaps, pores and more ill defined discontinuities. Using SEM, large pores have been identified in the heads of *Drosera* tentacles (Williams and Pickard 1969, 1974). These are sections where both the cuticularized layer and cutinized layer are absent (Juniper *et al.* 1989) and the cell wall is exposed to the exterior (Ragetli *et al.* 1972, Joel and Juniper 1982). In other carnivorous plants, pores are hard to observe using SEM because extensions of the cell wall project to the surface of the leaf (Juniper *et al.* 1989). These gaps are invisible under SEM because they are filled by permeable wall material and are referred to as cuticular gaps (Joel and Juniper 1982). The cuticularized layer in these cells is often thin or absent (Joel and Juniper 1982). Joel and Juniper (1982) suggest several ways in which cuticular gaps may form. These include the selective deposition and breakdown of cutin. But the mechanism in *Drosopyllum* is thought to be the tearing of the cutin layer through cell wall extension, which requires no special control mechanisms and can be due to normal cell growth (Joel and Juniper 1982). Joel and Juniper (1982) found that the rapid uptake of a water-soluble dye (neutral red) is a reliable indicator of cuticular

gaps and there is a correlation between the stainability and the presence of cuticular gaps. They also showed that immature *Drosophyllum* leaves could not absorb the dye neutral red because a lack of cell wall extension had allowed the cuticle to maintain its integrity. In contrast, mature leaves absorbed the dye due to tears in their cuticles caused by cellular extensions.

In this study, I use neutral red as an indicator of cuticular gaps in the plant *Roridula* to determine whether dissolved compounds lying on the leaf surface can be rapidly absorbed into the leaves of *Roridula*. I also use TEM to obtain direct evidence of cuticular gaps. The presence of cuticular gaps and the rapid uptake of neutral red should support the hypothesis of Ellis and Midgley (1996) who suggest that *Roridula* can absorb nitrogenous hemipteran faeces from their leaf surfaces. It would support the hypothesis that a mutualism exists between the carnivorous hemipteran *Pameridea* and the insect trapping plant *Roridula*. Finally, the presence of cuticular gaps may be regarded as a further adaptation to carnivory.

METHODS

The leaves of *R. dentata* and *R. gorgonias* were partially submerged in a 1% solution of neutral red for two minutes (See Juniper and Joel 1982). Both mature leaves and leaves in bud were treated in this manner (n=5 per species). In addition, the following three species of co-occurring non-carnivorous plant were treated in the same manner: *Saltera sarcocolla*, *Nivenia stokoei* and *Tetraria thermalis*. After 2 minutes of submersion, the leaves were removed from the solution and rinsed off. Transverse sections were made from neutral red absorbent leaves and these were examined under light microscope with no additional stains.

Leaf material was examined with a TEM using the methods of Joel and Juniper (1982). Leaves were fixed in 3% glutaraldehyde (in caccodylate buffer pH 7.2, 0.01M) for 5 hours.. The material was then postfixed in 1% OsO4 (in the same buffer) for 2 hours, dehydrated in an ethanol series, stained in uranyl acetate, and embedded in Spurr's resin (Spurr 1969). The material was sectioned using a Reichert ultracut S microtome and the sections were stained in lead citrate (Reynolds 1963). The material was photographed in a Zeiss EM109 electron microscope. Both mature and immature leaf material was examined from both *Roridula* species. Leaf material was also examined from *Saltera*.

Figure 1.
The following references apply to all the figures: **C** - cuticle, **EP** - Epidermis, **EW** - Epicuticular wax, **Ph** - phloem, **M** - mesophyll, **Scl** - sclerenchyma, **W** - cell wall, **X** - xylem

A. *Roridula dentata* leaf after being immersed in neutral red for two minutes.

B. Patchwork of absorptive and non-absorptive cells on the leaf of *R. dentata*.

C. Transverse section of a *R. dentata* leaf showing that most epidermal cells are capable of absorption.

D. Transverse section of a *R. dentata* leaf showing that neutral red rapidly reaches scherenchyma and mesophyll.

E. TEM of *Saltera* epidermal cell.

F. TEM of mature *R. dentata* epidermal cell showing cuticular pore.

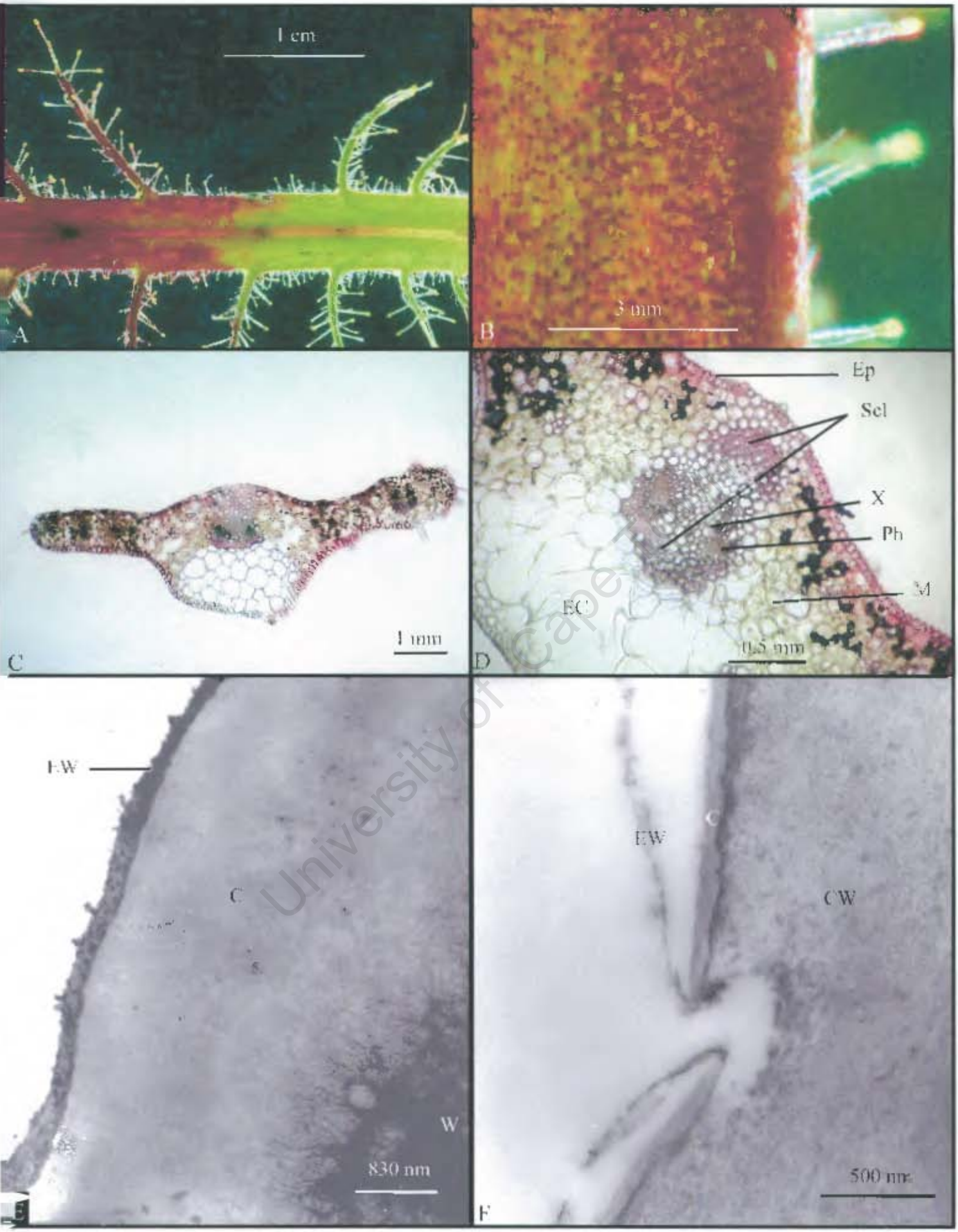


Figure 1.
The following references apply to all the figures: **C** - cuticle, **EP** - Epidermis, **EW** - Epicuticular wax, **Ph** - phloem, **M** - mesophyll, **Scl** - sclerenchyma, **W** - cell wall, **X** - xylem

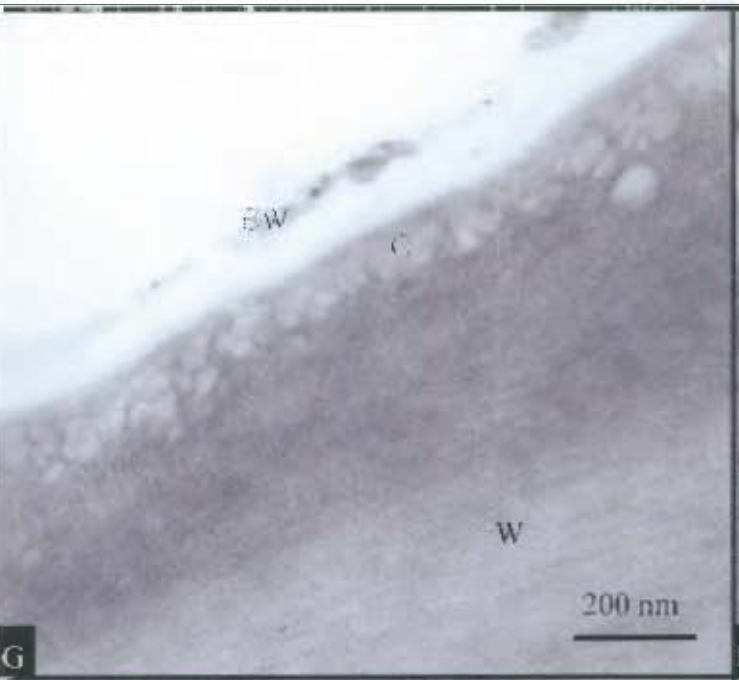
G. TEM of mature *R. dentata* epidermal cell showing reticulated wall elements reaching to the cell surface.

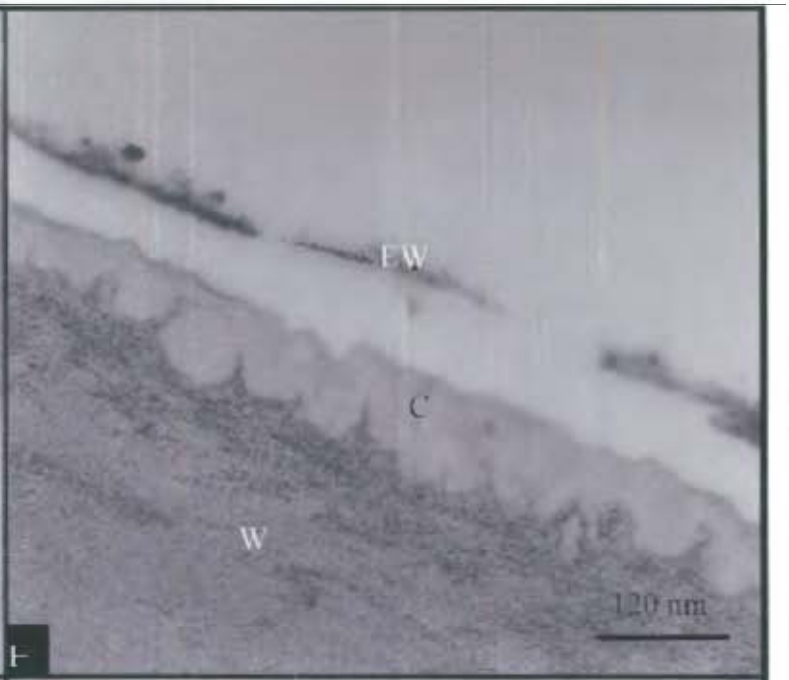
H. TEM of mature *R. dentata* epidermal cell showing cell wall elements extending towards the leaf surface.

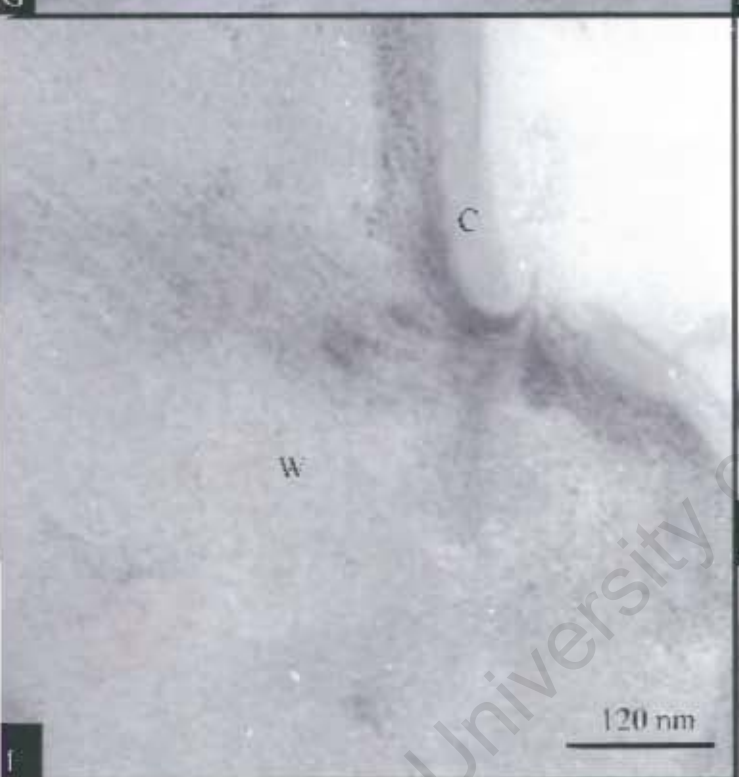
I. TEM of mature *R. dentata* epidermal cell showing cuticular gap with wall elements extending to the leaf surface.

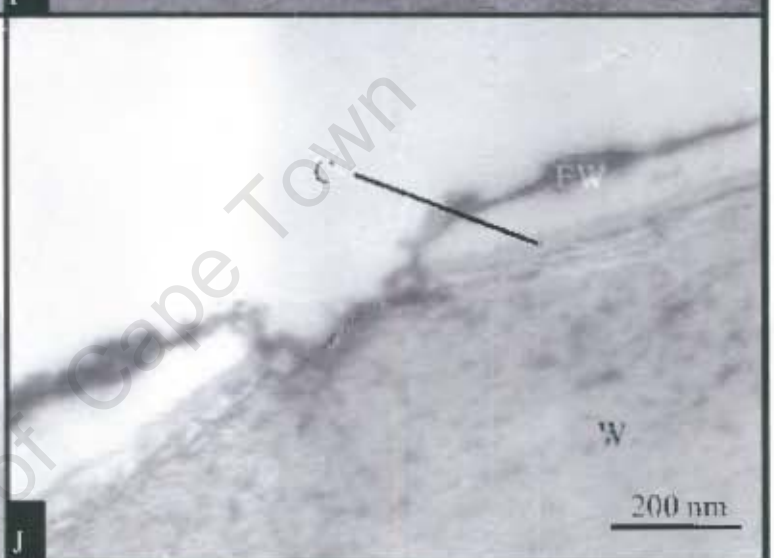
J. TEM of mature *R. dentata* epidermal cell showing a cuticular pore with a discontinuous cuticle

K. TEM of mature *R. dentata* epidermal cell showing cuticular gap.









RESULTS

The mature leaves of both *R. dentata* and *R. gorgonias* are permeable to neutral red following two minutes of immersion. The leaves show a distinct discontinuity where they were immersed in neutral red on one side (left hand side, Fig1a) and had no contact with neutral red (right hand side, Fig. 1a). In contrast, the immature leaves of *Roridula* and of co-occurring non-carnivorous plants are impermeable, suggesting a lack of cuticular discontinuities. The dye was taken up almost uniformly by the entire leaf surface of mature *Roridula*, barring some small patches of cells (Fig.1b). Transverse sections of *Roridula* leaves also indicate that dye was taken up by the majority of epidermal cells (Fig.1c, 1d). Mesophyll cells adjacent to the epidermis and sclerenchyma surrounding the vascular bundles both showed that they had taken up large quantities of neutral red (Fig. 1c, 1d). The continuous absorptive surface in *Roridula* contrasts to the specificity of absorption in *Drosophyllum* that only absorbs neutral red into a comparatively small number of specialized digestive cells (see Joel and Juniper 1982). TEM indicates that that the epicuticular wax layer is extremely thin (0-120 nm) and diffuse with many gaps that make it porous, it also seems very loosely attached to the underlying layers (Figs.1f, 1g, 1h, 1j). In contrast, the wax layer on non-carnivorous plants was comparatively thick (300-400 nm) and continuous (Fig. 1e). Similarly the cuticle of *Roridula* is extremely thin (0-120 nm) in comparison to non-carnivorous plants such as *Saltera* (Fig. 1e vs. Figs.1f-1k) that has a cuticle of between 2400 and 4000 nm thick. The epidermis of *R. dentata* and *R. gorgonias* leaves (mature and immature) both commonly showed invaginations of the cell wall (Fig. 1h) and these extended close to the leaf surface although they seldom broke through the cuticle such as in 1g). Holes, tears and pores were frequently observed in the cuticles of mature *Roridula* epidermal cells that allowed permeable cell walls to be in contact with the outside (Figs. 1f, 1l, 1j, 1k). The pore in Fig. 1k seems to have a plug of either cuticular wax or other external detritus and hence it is uncertain whether it would be permeable to liquid compounds.

DISCUSSION

The cell walls of *Roridula* leaves are often highly invaginated and reticulated in places and this is likely to create aqueous pathways between the inside and outside of the leaf. These invaginations are found in both old and young leaves and seldom reach the leaf surface. Since young leaves are not as permeable as older leaves, the primary cause of high cuticular permeability in *Roridula* leaves is most likely the presence of cuticular gaps and pores. Kersiens (1996) suggests that the existence of wide pores or cracks in the cuticle could increase cuticular permeance even if

they occupy a very small proportion of the leaf surface area. Joel and Juniper (1982) postulate that tearing can take place with normal cell growth.

The extensive staining by neutral red in *Roridula* suggests that the majority of epidermal cells are highly absorptive and that the entire epidermis has an absorptive function. This may be similar to the bottom zone of the pitcher plant, *Sarracenia*, where the entire inner leaf surface seems to be highly absorptive (Joel and Heide-Jørgensen 1985). In contrast only a few specialized digestive cells in *Drosophyllum* have cuticular gaps and are capable of rapid neutral red absorption (Joel and Juniper 1982). In the light of the spectacular neutral red absorption, the foliar absorption of nitrogen by *Roridula* is likely to be particularly efficient.

These results lend credence to the results of Ellis and Midgley (1996) who show that nitrogen from trapped flies is very rapidly absorbed by *Roridula* plants. Their results show that substantial amounts of fly nitrogen are incorporated into plant leaves after only 72 hours of capture. They postulate that such rapid nitrogen incorporation can only take place if digestion is immediate. The presence of cuticular gaps and pores over the entire epidermis provides the necessary absorptive structure for the rapid absorption of nitrogenous compounds on the leaf surface. These are the first data to suggest that *Roridula* has cuticular gaps similar to other carnivorous plants that would make the rapid nitrogen uptake postulated by Ellis and Midgley (1996) possible.

Lastly, I hypothesise that cuticular gaps in *Roridula* plants are another primary adaptation to carnivory suggesting that *Roridula* may be regarded as a carnivorous plant. Givnish (1989) defines carnivorous plants as those that have primary adaptations for prey attraction , prey capture and absorption. Many carnivorous plants only satisfy two of these criteria for example *Darlingtonia*, *Heliamphora* and *Sarracenia purpurea* do not produce digestive enzymes and are thought to digest prey with the aid of both micro and macro organisms. *Catopsis* is another carnivorous plant that passively traps prey and does not attract prey items or digest them. I regard the hemipterans living on *Roridula* as highly specific, obligate mutualists that act as functional digestive glands. *Roridula* traps large amounts of prey, it has the means to digest this prey and rapidly absorb the contents.

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PART 2

THE CONFLICT OF MUTUALISM

4 Conflict within the mutualism between *Roridula* and *Pameridea*

ABSTRACT

Unpredictability of reward quality/quantity is a major factor constraining the evolution of obligate one-on-one mutualisms. I examine unpredictability in the reward quality of an obligate, one-on-one mutualism between a carnivorous plant (*Roridula*) and a hemipteran (*Pameridea*) that facilitates prey digestion. In the field, I documented the densities of *Pameridea* on *Roridula* after a fire. In the greenhouse, I manipulated the hemipteran densities on *Roridula* and measured the mean relative growth rates of plants with differing hemipteran densities. Plant growth rates were correlated with hemipteran densities and plants with no hemipterans had negative growth rates. This suggests that hemipterans are important in facilitating nitrogen absorption. Plants with intermediate hemipteran densities had positive growth rates but growth rates were negative under very high hemipteran densities. I attribute this to the fact that hemipterans also suck plant sap and thus a trade-off exists. However, the benefits of facilitated nitrogen absorption outweigh the small losses incurred by sap sucking because the hemipteran densities seldom reach parasitic levels in the field. These observations highlight the exploitative nature of mutualisms and suggest that this mutualism may have evolved from a parasitism.

INTRODUCTION

Close one-on-one mutualisms are rare in nature (Howe 1984, Waser *et al.* 1996, Kearns *et al.* 1998) and anomalous in theory (Howe 1984). This general lack of specificity in mutualisms is thought to limit the potential coevolution because selective regimes may be diffuse where multiple partners coexist (Pellmyr *et al.* 1997, Hoeksema and Bruna 2000). However the geographical mosaic theory predicts that diffuse or non-specific mutualisms can coevolve in local pockets within the regional extent of the mutualism (Thompson 1994, Thompson 1999). Nevertheless, the evolution of specificity, intimacy and interdependence in mutualisms is largely determined by the predictability (e.g. is reward quality/quantity constant or does it fluctuate over time?) of relationships (Thompson 1994), and diffuse mutualisms may be less predictable than species-specific mutualisms. But almost all mutualisms have elements of unpredictability (some more than others) because so called "mutualisms" are thought to be reciprocal exploitations that provide net benefits to each partner (Maynard Smith and Szathmary 1995, Doebeli

and Knowlton 1998). The outcomes of so-called mutualisms are often context dependent, oscillating between parasitism and mutualism with changing biotic and abiotic factors, space and time (Howe 1984, Schemske and Horwitz 1988, Thompson 1988, Gaume *et al.* 1998, Thompson 1994). Changes in context (e.g. environmental conditions) may dictate whether a relationship is parasitic, commensalistic or mutualistic.

I examine the predictability of reward quality to the host plant in a highly specific digestive mutualism between a semi carnivorous plant (*Roridula*) and a hemipteran (*Pameridea*). *Roridula* is genus that catches large numbers of insect prey using sticky droplets on its leaves (Marloth 1903). The genus does not have digestive enzymes (Marloth 1910, Lloyd 1934, Ellis and Midgley 1996) and plants are reliant on *Pameridea* to facilitate the digestive process (Ellis and Midgley 1996). *Pameridea* is a genus of Miridae, specific to *Roridula* (Dolling and Palmer 1991). Unlike other insects, *Pameridea* is able to walk over the sticky traps of *Roridula* without being captured (Marloth 1903, Dolling and Palmer 1991). They consume the prey caught by *Roridula* and defecate on the plants' leaves. *Roridula* absorbs the faecal nitrogen directly through its very thin cuticle and can gain up to 70% of its nitrogen in this manner (Anderson and Midgley 2002). However I have observed that *Pameridea* apparently suck sap from *Roridula* plants and thus there may be a trade off between facilitated nitrogen absorption and sap-sucking. In addition to *Pameridea*, spiders also live on most *R. dentata* populations. The spiders (*Synaema*) affect the hemipteran densities (and thus the amount of insect nitrogen in *Roridula*) by preying on them and competing for resources (Anderson and Midgley, 2002).

Roridula plants grow within the fynbos biome, which is characterised by frequent fires (Cowling *et al.* 1992). They do not resprout after fire and regenerate as seedlings approximately six months after being burnt (pers. obs.). Thus, if all plants within a population are burnt, then hemipterans and spiders must recolonize from other populations. Alternatively, if some plants with their associated invertebrates survive the fire, then the invertebrates go through a bottleneck and must recolonize the seedling population from surviving plants (i.e. hemipteran densities are subject to fluctuations). I examine the stability in reward quality offered by *Pameridea* after manipulating the hemipteran numbers on *Roridula* cuttings in the greenhouse. Since unpredictable reward quality constrains the evolution of obligate, one-on-one mutualisms (and this is an obligate, one-on-one mutualism), reward quality in this mutualism should be predictable, and changes in hemipteran density should not have extremely negative effects on the outcome of the mutualism.

METHODS

To determine the natural abundances of *Pameridea* in the field, I examined their densities in a population before and after it had burned. My objective was to ascertain what hemipteran densities were realistic for use in the greenhouse. In November 1999, a *R. dentata* population (Pop8, 33° 22' S, 19° 15' E) burned down leaving six surviving plants. I had previously (February 1999) collected data on the approximate leaf biomass of *Roridula* and density of hemipterans at this site. I returned to this study site in February 2000, 2001 and 2002 to continue collecting plant biomass and hemipteran density data to plot their recoveries after fire. Plant biomass was calculated by estimating the total number of plants in the population, counting the numbers of terminal leaf clusters on ten plants and determining the dry mass of ten terminal leaf clusters. The vast majority of plants within the study site are the same age and hence their sizes were very similar. Forty terminal leaf clusters (on 40 different plants) were chosen where each terminal cluster was consisted of approximately 30 leaves (or 0.5 g, dry mass). These clusters were rapidly bagged and the adult hemipterans and spiders in each bag were counted. In addition, all hemipterans (juvenile and adult) from ten bags were brought back to the laboratory. They were frozen and then dried in a drying oven, after which the total hemipteran dry mass per leaf cluster was calculated. For every gram of adult hemipteran weighed, there were approximately 1.5 grams of juvenile hemipteran. Thus, by counting the numbers of adult hemipterans on each leaf cluster, I was able to estimate the dry mass of hemipterans (adults and juveniles) per leaf cluster without killing large numbers of them.

To determine whether the rewards offered by hemipterans depended on their numbers, I manipulated their densities on *Roridula* cuttings. In the greenhouse, cuttings of *Roridula* were weighed to the nearest milligram and placed in a 1/4 strength Hoaglands (Hewitt, 1966) solution lacking nitrogen and phosphorus. At the start of the experiment, each cutting had three buds and weighed between 0.950 and 2.500 g. The transition between woody stem and green leaf material was marked with a permanent pen. The aerial parts of the plants were enclosed in a fine meshed cage to stop insect movement to and from the plants. Four treatments (n=8) included a control (no *Pameridea* and no insect food), no *Pameridea* but plants fed with one fly per week, medium densities of *Pameridea* (3 adults per plant) and one fly per week, high densities of *Pameridea* (20 per plant) and one fly per week. After four months of growth, plants were removed and mean relative growth rate was calculated (Beadle 1987). To calculate this, I used only green leaf material and new woody material (i.e. old woody stem material present at the start of the experiment was subtracted from the total plant biomass). I repeated the experiments using *Roridula* and *Pameridea* from two different populations (Pop15,

Pop18, for approximate geographical positions, see figure 1, chapter 8). Treatments were copmpared using a Kruskal-Wallis test and post-hoc comparisons of mean ranks of all pairs of groups were made according to Siegel and Castellan (1998).

RESULTS

The intermediate numbers of hemipterans (three per cutting) used in the laboratory experiment were slightly higher than the highest average densities observed in the field during any year (Fig. 1). The maximum hemipteran densities per rosette (i.e. error bars) in 1999 and 2002 were higher than the intermediate densities used in the laboratory experiment. In contrast, the high hemipteran densities (20 per cutting) used in the laboratory were not close to the hemipteran densities observed in the field. From my extensive observations in the field, I predict that it is unlikely that such high hemipteran densities are naturally attained.

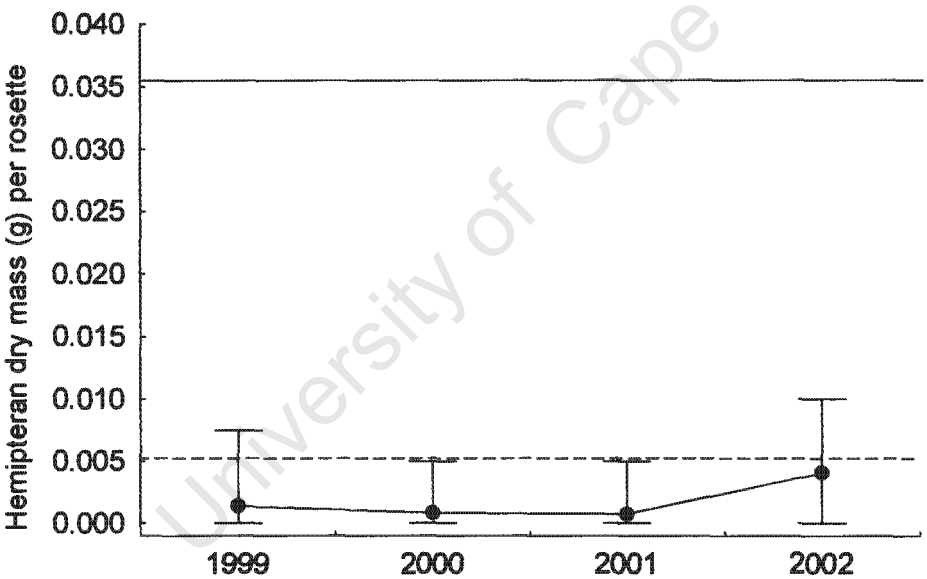

Figure 1. The dry mass of hemipterans (adults + juveniles) per rosette in a field population over four years with a fire between 1999 and 2000. Solid line represents hemipteran dry mass per rosette used in the laboratory for the high-density treatment. Dotted line represents the hemipteran density used in the laboratory treatment with intermediate hemipteran density. Whiskers are maxima and minima.

In the laboratory, plants from Pop18 with intermediate hemipteran densities had significantly faster growth rates than all other treatments (Fig. 2). In Pop18, plants with no hemiterans and no extra nutrition (control) had negative growth rates (median = -0.004 $g.d^{-1}$, a decrease in approximately 42 % over three months). The growth rates of the second treatment (plants fed with flies but that had no

hemipterans) were also negative and statistically no different from the control (median = -0.005 $g.d^{-1}$, a decrease of approximately 52 % over three months). Plants that had very high densities of hemipterans also had negative growth rates (median = -0.001 $g.d^{-1}$, a decrease of approximately 10 % over three months). The only treatment with a positive growth rate was when intermediate numbers of hemipterans were placed on *Roridula* (median = 0.006 $g.d^{-1}$). This translates to an increase of approximately 63 % over the three-month period. Differences between the treatments were statistically different (Kruskal-Wallis, χ^2 = 14.66, P = 0.0021). Variation around the median was larger for the three treatments with negative growth rates than in the treatment with intermediate numbers of hemipterans (Fig. 2).

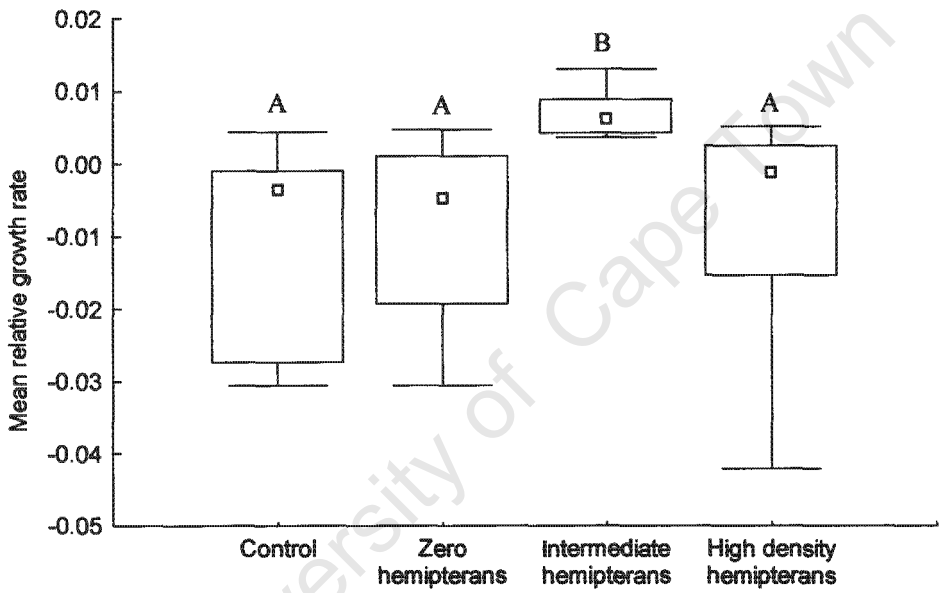

Figure 2. Mean relative growth rate of *Roridula* cuttings with different hemipteran densities (population 18). Control treatment had zero hemipterans and plants were not fed with flies. All other treatments were supplied with flies to supplement nitrogen and phosphorus. Data points are medians, boxes are 25 % - 75 % percentiles and whiskers are minima and maxima. Post hoc comparisons are significant at P < 0.05.

The same general trend (χ^2 = 9.2, P = 0.025) was also observed for hemipterans and plants from the site Pop15 (Fig. 3). The only positive growth rates were observed in the treatment with intermediate numbers of hemipterans (median = 0.002 $g.d^{-1}$, approximately 21 % increase over three months). All other treatments had growth rates between zero and -0.005 $g.d^{-1}$ (a maximum decrease of 52 % after three months). However in this experiment, the only significant difference between treatments was between plants with intermediate hemipteran densities and those with high hemipteran densities (P = 0.023). Lack of statistical power meant that unlike in Population 18, the intermediate hemipteran density treatment was not

significantly different from the first two treatments (P > 0.05). As with the experiment using population 18, the first two treatments had the same growth rates as the last treatment (P < 0.05).

It is important to note that plant growth rates are used as a surrogate for fitness. It assumes that larger plants produce more offspring than smaller plants.

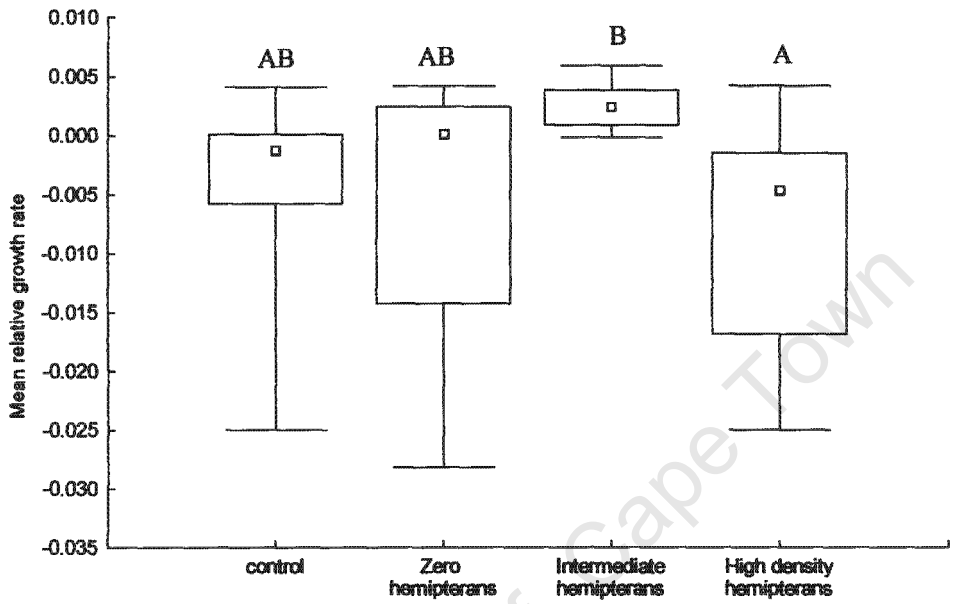

Figure 3. Mean relative growth rate of *Roridula* cuttings with different hemipteran densities (population 15). See fig. 2 for treatment details.

DISCUSSION

The greenhouse study suggests that variation in hemipteran densities can potentially affect growth rates in *Roridula* plants. Bronstein (1994) predicts that the net outcomes of mutualims are frequently dependent on the abundance of partners. She states that "per capita benefits often increase as mutualist populations grow but then shift downwards again at large population sizes." This is precisely what the results of this study (particularly the experiment using population 18, Fig. 2) suggest: At very high densities hemipterans may be detrimental to *Roridula* (probably due to sap-sucking by hemipterans). This is because the cost of sap sucking out-weighs the benefits of facilitated nitrogen absorption at high hemipteran densities. In contrast, at intermediate hemipteran densities, the cost of sap sucking is out-weighed by the benefits of facilitated nitrogen absorption. Since hemipteran numbers do not seem to reach parasitic levels in the field, the relationship is likely to be predominantly mutualistic. Plants that were fed with flies and had no hemipterans, had the same growth rates as control plants where no flies were fed to

plants. This suggests that the presence or absence of hemipterans is important in facilitating the nitrogen absorption process, corroborating the results of Ellis and Midgley (1996). Lower variation was observed within treatments having intermediate numbers of hemipterans than all other treatments. This can be attributed to the fact that individual variations in fitness are more apparent under highly stressful conditions than they are under optimal conditions.

This experiment was conducted under laboratory conditions where nitrogen and phosphorus were only given to plants in the form of flies on their leaves. In reality, this situation is unlikely to exist in nature, as soil nitrogen and phosphorus are present. However, soil conditions in fynbos are characteristically nutrient poor (Stock and Allsopp 1992) and probably poorer still in the leached habitats typically occupied by carnivorous plants (Givnish 1984, 1989). Furthermore, *Roridula* has a very poorly developed root system (Obermeyer 1970), which is unlikely to make *Roridula* a strong competitor for the few soil nutrients available. Although *Roridula* most probably obtains some nitrogen or phosphorus through its root system (Obermeyer 1970), the amounts received are likely to be minimal and therefore I can justify using a nutrient solution with no nitrogen or phosphorus.

Previously, field experiments have demonstrated that indirect measures of *Roridula* fitness ($\delta^{15}N$) are positively associated with the presence of hemipterans (Ellis and Midgley 1996, Anderson and Midgley 2002). However, these studies on *Roridula* nutrition did not show that the presence or absence of hemipterans could actually affect plant growth. This is the first study to show that variability in hemipteran densities can influence plant growth. Furthermore, this is also the first study to show that very high hemipteran densities may have negative effects on *Roridula* growth rates. This study also creates some uncertainty surrounding the role of species-specific spiders on *R. dentata*. Anderson and Midgley (2002) demonstrated that the species-specific spider (which only occurs on *R. dentata*) negatively affects hemipteran densities by consuming them and also by competing for resources. They also showed that populations with low hemipteran densities were not as efficient at obtaining nitrogen from hemipterans. Consequently, they regarded spiders as kleptoparasites because they effectively "steal" nitrogen from *Roridula* by lowering hemipteran densities. However, this study shows that very high hemipteran densities may at times be detrimental to *Roridula* and that the relationship is optimal for *Roridula* under intermediate hemipteran densities. Thus, it is possible that spiders provide a "service" to plants by keeping hemipterans at an optimal level. More work needs to be done in natural populations, examining the role of spiders in the *Roridula-Pamerideа* mutualism.

Unpredictability in reward quality/quantity is observed in laboratory conditions although indications are that hemipteran numbers are seldom high enough in the field to reach parasitic levels. This may partially explain how the mutualism was able to evolve. Sap-sucking may also be a relict of a parasitic past and this may have facilitated the evolution of high specificity and obligacy. In contrast to mutualisms, species-specific parasitic relationships (especially insect-plant relationships) are very common and are perhaps the norm (Thompson 1994). The selective pressures on phytophagous insects are sometimes so strong that they have been found to specialize at the micro level, forming demes adapted to individual plant populations (Fox and Morrow 1981, Karban 1989, Mopper *et al.* 1995). Models have predicted that once an element of specificity is present in a relationship, then evolution is likely to continue in this direction (Law and Koptour 1986). Thus, if a mutualism is able to evolve from a species-specific parasitism, it is most likely to remain specific or perhaps become even more specific. With parasitism as a starting point, the problem of first infection and the evolution of specificity may be taken care of and one only needs to consider how the parasitism could change to mutualism (Boucher *et al.* 1982).

The evolution of the mutualism between Pameridea *and* Roridula

Pameridea belong to the predominantly phytophagous family Miridae which are often very specific or monophagous (Schuh 1995). However carnivory is thought to have evolved several times within the herbivorous Miridae (China 1953). China (1953) suggested, that phytophagous insects on carnivorous plants may easily evolve carnivory by supplementing their diets with insects caught by the sticky plants that they inhabit. It is possible that *Pameridea* started as a species-specific sap-sucking insect on *Roridula*, a plant with sticky hairs for defence. *Pameridea* may have undergone a diet change and switched to eating small insects that were incidentally captured by the sticky hairs of *Roridula*. Only after *Pameridea's* diet change from sap to insects would it have been possible for *Roridula* to gain benefits from the association and it would have been at this stage that the plant would have evolved more effective traps. These would have been designed to trap insects rather than deter them. Given the above scenario it is possible for this mutualism to have developed and evolved from a previously parasitic relationship. This could explain the high degree of species-specificity observed in this system. However, a phylogenetic study of the Miridae is needed to show when carnivory evolved in *Pameridea*. This may indicate whether carnivory evolved before the association with *Roridula* or if it evolved as a response to the association with *Roridula*. Unfortunately no such phylogeny is presently available.

The evolution of the species-specific mutualism between *Roridula* and *Pameridea* is most probably similar to that of other species-specific mutualisms. The most celebrated of these include figs and fig wasps, yuccas and yucca moths, and globeflowers and their pollinators. The common thread binding these three mutualisms is that they are all thought to have evolved from host-specific parasitic relationships (Figs-Addicott *et al.* 1990, globeflowers-Pellmyr 1989, yuccas- Davis 1967). All of the insects are host-specific seed predators that have become host-specific pollinators. A trade off exists between the pollinators and plants because pollinators still oviposit on and destroy a proportion of seeds produced by the plants. The relationship between *Roridula* and *Pameridea* is different from other host-specific mutualisms because *Pameridea* are not seed predators. However, like the other host-specific mutualisms, *Pameridea* has a very intimate relationship with its host (i.e. it spends most of its life on *Roridula*), it shows relicts of a parasitic past, it oviposits in *Roridula* (pers. obs.) and has been implicated in the pollination of *Roridula* (Marloth 1903, 1925, chapter 1).

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PART 3

THE EXPLOITATION OF MUTUALISMS

5 It takes two to tango but three is a tangle: The effect of exploiters in the *Roridula-Pameridea* mutualism

ABSTRACT

***Roridula dentata* is associated with hemipterans, which facilitate nitrogen assimilation from insects. *Roridula dentata* is also associated with spiders and their role in digestion is unknown. I quantified approximately how much nitrogen *Roridula* assimilates from insects through "indirect digestion." Using $\delta^{15}N$ I then determined whether nitrogen absorption from hemipteran insects differs with varying spider densities. In this way, I was able to determine their nutritional role. At low spider densities, indirect digestion of prey accounts for approximately 70% of plant nitrogen. These values are comparable to methods of direct prey digestion found in other carnivorous plants. However spiders decrease the numbers of hemipteran individuals inhabiting *Roridula* plants and also decrease efficiency of indirect prey digestion by up to 30%. Spiders seem to be exploiters as they exploit plant rewards without offering any rewards in return. Their effects are similar to cheaters. Indirect carnivory is still efficient enough when hemipteran densities are at their lowest, ensuring that the mutualism does not break down.**

INTRODUCTION

Exploitation is an inevitable part of almost every mutualistic system (Springer and Smith-Vaniz 1972; Janzen 1975; Thompson 1982, Bronstein 2001). The invasion of mutualisms by exploiters/cheaters may cause variation in the reward quality of mutualisms (e.g. Pellmyr *et al.* 1996). Exploiters or cheaters are organisms that "obtain commodities offered by mutualists but that fail to reciprocate by offering commodities in return (Bronsten 2001)." For example, exploiters such as nectar thieves may leave little rewards for mutualistic pollinators. As a result, these pollinators may be forced to seek flowers with more reliable rewards (Soberon and Martinez del Rio 1995). The result may be mutualism collapse, which may trigger the extinction of obligate partners (Boucher *et al.* 1982), although there is no data to support this. This fluctuation of reward quality (e.g. nectar availability) has also been identified as one of the major factors constraining the evolution of specialist or one-on-one, obligate mutualisms (Thompson 1994). Howe (1984) suggests that diverse faunas frequently affect reward quality negatively by competing with mutualists. As a result, diverse faunas are sometimes viewed as a source of unpredictable reward

quality (see Bronstein 1994). Despite the fact that exploitation may affect the stability of mutualisms, the theoretical implications of exploitation have not been well explored (Bronstein 2001). Some new conceptual and theoretical models oppose conventional theory and suggest that obligate exploiters and mutualists can often coexist without exploiters driving the mutualism to extinction (Bronstein 2001, Yu *et al.* 2001 and Yu 2001), however no experimental data exists to support either hypothesis. Bronstein (2001), Yu *et al.* (2001) and Yu (2001), explain how mutualists and exploiters can coexist however they do not offer explanations of how they evolve together from the start. It is possible that exploiters can drive potential mutualisms to extinction before they have had time to evolve a great degree of stability.

Carnivorous plants have a plethora of closely associated invertebrate fauna with both facultative and obligate relationships that range from parasitisms to mutualisms (Beaver 1983; Beaver 1985; Juniper *et al.* 1989). Because of their diversity, these systems may be excellent for exploring the evolution and breakdown of mutualisms and the role of exploiters. However very few studies have examined carnivorous plant relationships in detail: Zamora (1990) observed that ants, which are kleptoparasites of *Pinguicula*, steal a large proportion of prey. Clarke and Kitching (1995) noted that *Nepenthes* pitchers may capture an excess of prey, which often causes putrification of the pitcher if not removed. But species-specific ants living on the *Nepenthes* plants remove excess prey, a process that reduces the incidence of putrification by up to 12 fold. Bradshaw and Creelman (1984) showed that pitcher fauna may increase the rate of digestion in *Sarracenia purpurea* and thus could be considered mutualists.

The plant *Roridula gorgonias* Planch. catches insects using sticky traps but has no digestive enzymes to utilise the prey (Marloth 1925). Using an isotopic labelling technique, Ellis and Midgley (1996), determined that *R. gorgonias* has a digestive mutualism with a species-specific hemipteran (Dolling and Palmer 1991) that consumes the captured prey. These hemipterans defecate on *Roridulas'* leaves and the plants absorb nitrogen directly through their cuticle (Ellis and Midgley 1996). However, the amount of nitrogen gained in this manner remains to be quantified.

Using isotopic methods, I aim to examine and quantify the effects of two different relationships with *Roridula*. I examine an unstudied species of *Roridula* (*R. dentata* L.) with a unique species of associated hemipteran (*Pameridea marlothi* Poppius). A monophyletic group of hemipterans (one genus, two species) is associated with every known *Roridula* population and each of the two *Roridula* species is associated with a different species of *Pameridea* (Dolling and Palmer 1991). In addition, a species-specific spider (*Synaema marlothi* Dahl., Thomsidae) is also associated exclusively with *R. dentata*. These spiders occur on most *Roridula dentata* populations in the southern and central part of their range. Where *Synaema* occurs, it is usually very

common and builds its nests in the axils of *Roridula's* branches and dead leaves (Marloth 1925; pers. obs.). The spiders are carnivores and presumably compete with hemipterans for resources (Marloth 1925). In addition it has been recorded that *Synaema* also consumes *Pameridea* (Lloyd 1934; pers. obs.). Spiders may thus disrupt the mutualism between *Roridula* and *Pameridea* if they decrease the numbers of hemipterans but do not supply plants with nitrogen. Alternatively spiders may also facilitate nitrogen absorption in a similar way to *Pameridea.* Until now, no studies have been performed on the spiders and their role in *Roridula* nutrition. Spiders are also very commonly associated with other carnivorous plants but their roles in other systems also remain unstudied. In this study, I quantify the nutritional benefits of the hemipteran mutualist and determine the nutritional role played by spiders.

METHODS

The densities of hemipterans and spiders may be related to nitrogen uptake in plants, therefore, these were the first data collected. In addition to the five sites mentioned below, density data were collected from another four sites. These sites were Pop22 (32° 07' S, 19° 00' E), Pop14 (32° 40' S, 19° 11' E), Pop13 (32° 50' S, 19° 12' E) and Pop12 (32° 49' S, 19° 13' E). *Synaema marlothi* occurred at all except three study sites (Pop22, Pop9, Pop13) used in this study. At every *R. dentata* population, 30 rosettes (approximately 20 leaves each) from random plants were rapidly bagged and the number of adult hemipterans and spiders were counted. This enabled us to observe the densities of hemipterans and spiders relative to each other. It also enabled us to relate levels of insect derived nitrogen in *Roridula* to hemipteran or spider density.

Three geographically close sites (Pop15: 32°39' S, 19° 11' E, Pop16: 32° 40' S, 19° 11' E, and Pop17: 32° 40' S, 19° 11' E) were used to investigate the nutritional impacts of hemipterans and the role played by spiders. These sites were chosen because they shared very similar vegetation, are geographically close and differed in the density of spiders and *Pameridea*. Young leaf samples (n = 5 per species) were collected from *Roridula* and reference plants at each site for isotopic analysis. Approximately one week before leaf harvesting, all leaves were bagged in transparent nylon bags while still in bud so as to exclude prey items and *Pameridea.* The following reference plants were collected at each site: *Stoebe plumosa* (L.) Thunb. (Asteraceae), *Metalasia densa* (Lam.) Karis (Asteraceae) and *Diosma sp.* (Rutaceae). Reference plants were chosen according to their mycorrhizal associations as this is thought to influence $\delta^{15}N$ values (Hobbie *et al.* 2000; Schmidt and Stewart 1997). Since *Roridula* has a vesicular-arbuscular mycorrhizal (VAM) association, reference plants were chosen that also had this association (based on Allsopp and Stock 1993). *Pameridea* (n = 5 from each population) and live prey items were also collected from

each population (prey items were pooled by grinding all insects together). Prey consisted of four Hymenoptera, three Coleoptera, three Diptera and one Hemipteran. At these sites I felt it unnecessary to control for rooting depth as *R. dentata* has a shallower rooting depth than all the other plants sampled. Since $\delta^{15}N$ typically increases with rooting depth (Schmidt and Stewart 1997; Gebauer and Schulze 1991; Hogberg 1997) any differences between *Roridula* and reference plant $\delta^{15}N$ values will thus be conservative.

In a separate experiment, two more sites were used to compare levels of insect derived nitrogen in *R. dentata* with and without spiders. These sites are geographically distant from Pop15, 16 and 17. The first population (Pop9) is a small *R. dentata* population with no spider inhabitants and is in the Tulbagh region (33° 21' S, 19°05' E). The closest known *R. dentata* population to this is situated approximately 17 km away in the Ceres area (33° 22' S, 19°15' E). This site (Pop8) is dry with sandy soil and has several plant species (e.g. *Leucadendron rubrum* Burm.f., and *Protea nana (P.J. Bergius) Thunb.*, both Proteaceae) in common with Pop9. In contrast to Pop9, the spider *Synaema marlothi* is abundant at Pop8. Leaves were bagged before sampling as above. At the time of sampling, five *Roridula* and five *Protea nana* (reference plants) were sampled per population.

Mass spectrometer methods

The relative contribution ($\%N_{CI}$) by insects to the nitrogen content of a carnivorous plant was calculated by Schulze *et al.* (1991) using methods modified from Peterson and Fry (1987). Where $\delta^{15}N_{CAR}$ is the $\delta^{15}N$ value for the carnivorous plant, $\delta^{15}N_I$ is the $\delta^{15}N$ value for insect prey and $\delta^{15}N_{REF}$ is the $\delta^{15}N$ value for reference plants (see equation 1).

$$\%N_{CI} = \delta^{15}N_{CAR} - \delta^{15}N_{REF}/\delta^{15}N_I - \delta^{15}N_{REF} \qquad 1$$

The equation used to determine the percentage of nitrogen that *Roridula* gains from hemipteran faeces is derived from Schulze *et al.* 1991 (see equation 2).

$$\%N_{RI} = \delta^{15}N_{ROR} - \delta^{15}N_{REF}/\delta^{15}N_{FAECES} - \delta^{15}N_{REF} \qquad 2$$

Two methods were used to calculate $\delta^{15}N_{FAECES}$. The first is an indirect method while the second is direct:

Method 1.

Since insects discriminate against ^{14}N during excretion (Peterson and Fry 1987) and

retain ^{15}N, their faeces will be depleted of ^{15}N, relative to their food source. The value of $\delta^{15}N$ retained by an insect is:

$$\delta^{15}N_{RETAINED} = \delta^{15}N_{INSECT} - \delta^{15}N_{PREY} \quad 3$$

The $\delta^{15}N$ value for faeces will be $\delta^{15}N$ of the prey minus the $\delta^{15}N$ retained by the insects:

$$\delta^{15}N_{FAECES} = \delta^{15}N_{PREY} - \delta^{15}N_{RETAINED} \quad 4$$

Method 2.

Ten hemipterans from each population were kept in separate petri dishes for three days. After this period, all hemipterans were dried and homogenised together. Their faeces were also left to dry and were scraped off the petri dishes. Both faeces and hemipterans were run on the mass spectrometer to determine average $\delta^{15}N$ values for faeces and hemipterans.

RESULTS

There is a significant (r^2 = 0.57 and p = 0.018) negative relationship between hemipteran density and spider density (Fig. 1). The highest hemipteran densities were found at the study site Pop22 that had no spiders. The site Pop9 also had no spiders but hemipteran densities were not as high as two other sites with very low spider densities. The highest spider and lowest hemipteran densities were found in the site Pop17.

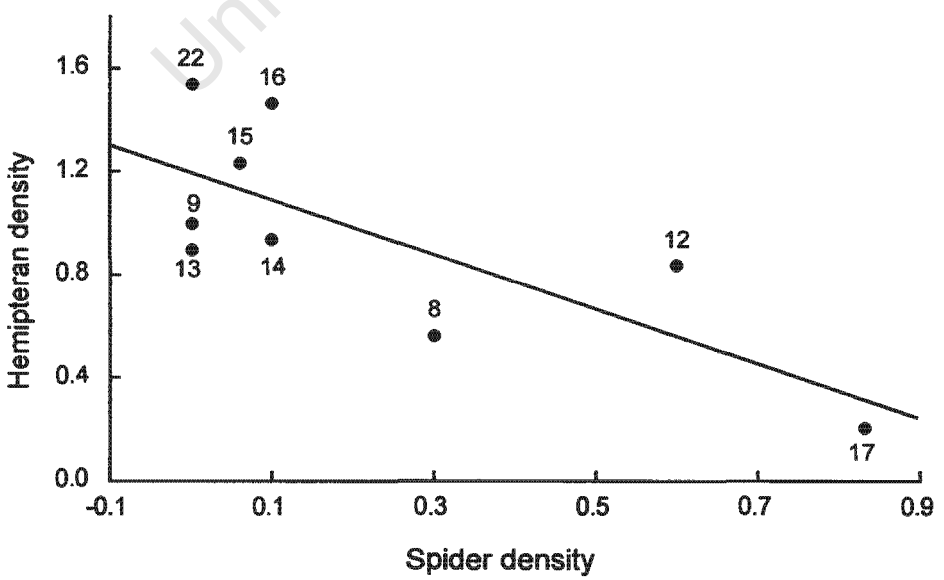

Figure 1. The densities of the hemipteran, *Pameridea* and spider, *Synaema marlothi* at a range of different study sites.

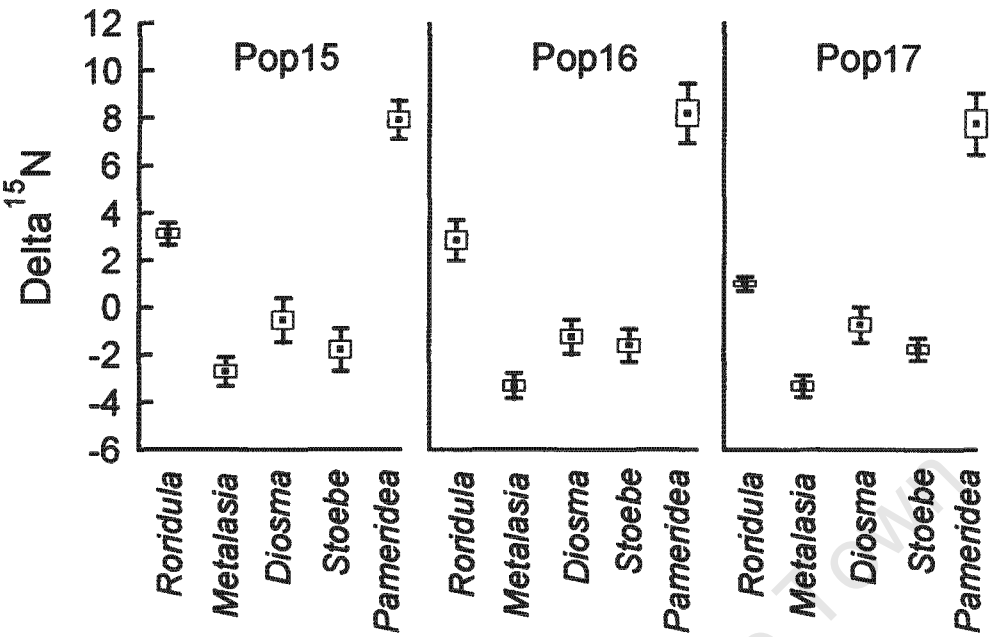

Figure 2. Delta ^{15}N values for *R. dentata*, reference plants and *Pameridea* at three study sites (Pop15, Pop16 and Pop17) within close proximity of each other. Mean, box = SE and error bars = 1.96 x SE

At sites Pop15, 16 and 17, there was no significant difference (Kruskal-Wallis, Fig. 2) between the δ^{15}N values for *Metalasia* (χ^2 = 3.75, p = 0.1534), *Diosma* (χ^2 = 1.33, p = 0.5134) and *Stoebe* (χ^2 = 1.33, p = 0.5134). In contrast, the δ^{15}N values of *Roridula* plants at the three sites were significantly different from each other (χ^2 = 6.92, p = 0.0308). Hemipteran densities at the three sites also differed significantly (χ^2 = 18.61, p = 0.0001). The lowest mean hemipteran densities (SE) were from Pop17 (0.20 ± 0.70). Hemipteran densities on Pop15 and Pop16 were 1.23 (0.22) and 1.46 (0.21) respectively. Using equation 2, the levels of insect nitrogen ($\%N_{RI}$) at each study site were calculated (using the indirect method - 1) as Pop15 = 70%, Pop16 = 71% and Pop17 = 41 % (Table 1). The $\delta^{15}N_{FAECE\ S}$ values were extremely similar using the direct and indirect approaches (Table 1).

Table 1. The percentage of nitrogen derived from hemipteran faeces for *Roridula* at three study sites, calculated using two methods. ^{15}N value for prey was 6.57 ‰.

Study site	δ^{15}N *Roridula* (SE)	Faecal δ^{15}N (Method 1)	Faecal N (Method 1)	Faecal δ^{15}N (Method 2)	Faecal N (Method 2)
Pop15	3.12 (0.31)	5.19	70 %	5.32	68 %
Pop16	2.86 (0.21)	4.90	71 %	4.98	70 %
Pop17	1.03 (0.13)	5.34	41 %	4.98	43 %

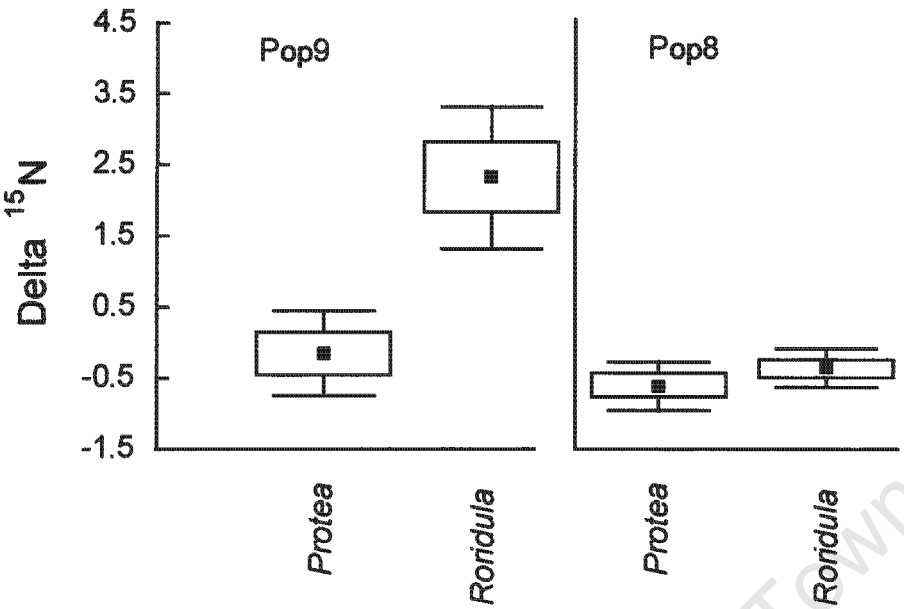

Figure 3. Delta ^{15}N values for *R. dentata* and reference plant at two different study sites: (Pop8 and Pop9). Sites differed in spider and hemipteran densities (see Fig. 1). Mean, box = SE and error bars = 1.96 x SE

Plants from the study site Pop8 had a mean of 0.3 ± 0.2 (SE) adult spiders per rosette. These plants had significantly fewer hemipterans (Mann-Whitney U, Z = -2.04, p = 0.042) than plants at the Pop9 site, which had no spiders. The δ^{15}N signatures of *P. nana* from the two sites were similar (Fig. 3). At Pop8, *R. dentata* had similar δ^{15}N values to the reference plant but at Pop9, *R. dentata* had very high δ^{15}N values (Fig. 3). *Roridula* plants from Pop8 had higher δ^{15}N values than all other plants tested in the study (Kruskall-Wallis, χ^2= 10.30, p = 0.016). Due to the lack of an appropriate (VAM) reference plant, I was not able to calculate the percentage of nitrogen derived from insects at Pop8 and Pop9. Although the comparison between Pop8 and Pop9 cannot be used on its own to show the role of spiders in *Roridula* nutrition, it nevertheless supports the other results in this study.

DISCUSSION

Spiders negatively influence the strength of the plant-hemipteran mutualism and thus the amount of insect derived nitrogen in *Roridula* varied according to spider densities. The majority of *Roridula* plants analysed fell within the range of insect nitrogen found by Schulze *et al.* (1991), where δ^{15}N = 0.819-3.302 ‰. However, at one site (Pop8), the δ^{15}N values were much lower (0.35 ± 0.13 ‰) and δ^{15}N was the same as for the

non-mycorrhizal reference plant. These comparatively low $\delta^{15}N$ values suggest that *Roridula* does not obtain much nitrogen from insects at this site.

At two sites (Pop15 and Pop16) *Roridula* was deriving large proportions of nitrogen ($\%N_{RI}$ = 70 %) from hemipteran faeces and therefore very little from the soil. Insect dependence in *Roridula* is much higher than the average for upright *Drosera* ($\%N_{DI}$ = 50 %) calculated by Schulze *et al.* (1991) and is one of the highest values of insect nitrogen uptake recorded. Insect dependence in *Roridula* is comparable to the most insect dependent carnivorous plants such as *Dionaea* (Schulze *et al.* 2001) and pitcher plants (Schulze *et al.* 1997). After fire, *Dionaea* obtains approximately 75 % of its nitrogen from insects. Pitcher plants such as *Nepenthes* and *Darlingtonia* can also be extremely insect dependent (61.5 % and 76.4 % respectively). Insect nitrogen in *Roridula* exceeds insect nitrogen in the pitcher plant *Cephalotus* (26 %). However, at a single site, (Pop17) *Roridula* obtained very little nitrogen from insects, although it was still similar to the average for *Drosera* calculated by Schulze *et al.* (1991).

Both $\%N_{RI}$ and ^{15}N values were variable, indicating that the nutritional benefits to *Roridula* is variable. At the sites Pop8 and Pop17, ^{15}N and $\%N_{RI}$ respectively were exceptionally low. These were also the only two sites analysed with high spider densities and low hemipteran densities. The low $\delta^{15}N$ values and low percentages of insect nitrogen at sites with high spider densities suggest that spiders do not contribute significant amounts of nitrogen to *Roridula* plants. Spiders can thus be regarded as exploiters. Differences in the behaviour of spiders and hemipterans may account for the differing effects of these two invertebrate taxa on plant nutrition. For example, spiders spend large amounts of time in nests and presumably this is where they defecate. Alternatively, they allow their faeces to fall to the ground. In both cases, nitrogen is not easily accessible to *Roridula*. This contrasts with hemipterans that defecate on the leaves of *Roridula*.

Insect derived nitrogen may be crucial for the survival of *Roridula* in the wild. In addition, hemipterans are major pollinators of *Roridula* and are responsible for up to 65 % of seed set in *R. dentata* (unpublished data). The combination of nutritional and pollination benefits of this mutualism indicates that hemipterans may be crucial for plant survival. I tentatively classify the relationship as a one-to-one, obligate mutualism, which Howe (1984) describes as "rare in practice and anomalous in theory." This raises the important question of how such a close, one-on-one mutualism evolved and how it persists in the face of exploitation?

The question may be partly answered by comparing levels of insect derived nitrogen in *Roridula* populations (with high rates of kleptoparasitism) against levels of insect

nitrogen in other carnivorous plants. Although the benefits of prey capture fluctuate, *Roridula* still receives a substantial amount of insect nitrogen (40%) even when spider densities are at their highest. This is still far higher than the levels of insect nitrogen found in *Drosera erythrorhiza* (Dixon *et al.* 1980). Furthermore, it is comparable with the average percentage of insect nitrogen (50 %) found in upright Drosera (Schulze *et al.* 1991). I speculate that for *Roridula*, the gains of mutualism outweigh the costs, even in the face of heavy exploitation. Thus it is unlikely that the present levels of exploitation should cause mutualism collapse in *Roridula*.

This study identifies an obligate, one-on-one mutualism between a carnivorous plant and a carnivorous hemipteran, making it one of the few obligate, one-on-one mutualisms known. In addition, it suggests that at a local scale, mutualists and exploiters can coexist, provided that the negative effects of exploiters are not too great. I also show that impacts of exploiting can vary spatially. Yu *et al.* (2001) suggests that spatial variation may explain the persistence of mutualists and exploiters. The results of Chapter 4 are also interesting in the light of this chapter: Chapter 4 suggests that *Pameridea* may be antagonistic to *Roridula* under very high densities. However, by having a negative impact on hemipteran densities, spiders may in fact provide stability to the system by keeping hemipteran densities at optimal levels. Thus, spiders may not be exploiters after all!

Other carnivorous plants such as *Sarracenia* and *Nepenthes* may also provide similar insights into mutualisms and exploitation. Like *Roridula*, many *Sarracenia* species do not have digestive enzymes (Juniper *et al.* 1989). In addition, there is a large, unstudied invertebrate fauna associated with these plants and I predict that many may have digestive mutualisms similar to those found in *Roridula*

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6 When did exploiters associate with the *Roridula-Pameridea* mutualism?

ABSTRACT

In addition to the species-specific mutualism with *Pameridea*, *Roridula* also has relationships with three non-mutualistic species of spider and another hemipteran. However, the evolution of species-specificity and obligacy in mutualisms may be opposed by the presence of other closely associated fauna, which tend to exploit existing mutualisms. The aim of this manuscript was to determine when the non-mutualists became associated with the *Roridula-Pameridea* mutualism. I document the phylogenetic and geographic associations between *Roridula* and *Pameridea* (two species each). I also overlay the distribution patterns of *Roridula* and the non-mutualistic species to see how closely they are correlated. Each sister species of *Pameridea* is associated with a different *Roridula* species and despite large disjunctions in *Roridula's* range, each *Roridula* population is associated with *Pameridea*. This suggests that *Roridula* was associated with *Pameridea* before fragmentation/vicariance events split the genus allowing the evolution of two separate species. In contrast *Roridula* is only associated with non-mutualists in parts of its current range because non-mutualists are not able to traverse the large gaps (disjunctions) in *Roridulas'* distribution. This suggests that non-mutualists only became associated with the mutualism after vicariance events fragmented *Roridula* populations. I hypothesise that *Pameridea* and *Roridula* were closely associated for a long period before the invasion of non-mutualists. The absence of associated non-mutualist species may have helped facilitate the evolution of obligacy between *Roridula* and *Pameridea*.

INTRODUCTION

When a mutualism evolves it may become subject to exploitation because the association often provides a (novel) resource, which other organisms can exploit (Thompson 1994, Bronstein 2001). These so called "exploiters" may affect existing mutualisms through competition, cheating or predation, and the accumulation of exploiters may ultimately cause the breakdown of mutualisms over evolutionary time (Thompson 1982). For example, nectar robbing by bees and birds caused legitimate pollinators (hummingbirds) to cease visiting the plant *Justica aurea* (McDade and Kinsman 1980). Exploitation and the indirect effects of diverse, associated faunas may also cause unpredictability in the reward quality/quantity for

mutualists and his may constrain the evolution of species-specific, obligate associations (Howe 1984, Thompson 1994). Diverse invertebrate faunas may also dilute strong directional selection pressures. This may result in a selective regime that is too diffuse for tight coevolution between any two species (Hoeksema and Bruna 2000). Unpredictability, mutualism breakdown and the low potential for coevolution in mutualisms are primary factors responsible for the paucity of species-specific, obligate mutualisms observed in nature (Thompson 1994). Consequently, highly specific mutualisms are sometimes thought to evolve in environments with low species diversity (Howe 1984) where interactions with additional species are minimized. Alternatively, highly specific mutualisms (e.g. fig-fig wasp, yucca-yucca moth, globe flowers and fly pollinators) may evolve from previously parasitic relationships (Herre 1989, Pellmyr 1989, Addicott *et al.* 1990, Powell 1992), which are commonly species-specific (Thompson 1994).

In this chapter, I document the fauna (and their distributions), which are frequently associated with the near carnivorous plant *Roridula* that has a species-specific digestive mutualism with a hemipteran (Dolling and Palmer 1991, Ellis and Midgley 1996). The plant family Roridulaceae is a monophyletic group consisting of a single genus and two species (Obermeyer 1970) endemic to South Africa. Based on molecular phylogeny, the genera most closely related to *Roridula* are from the family Sarraceniaceae and include the carnivorous genera *Darlingtonia*, *Heliamphora* and *Sarracenia* (Bayer *et al.* 1996, Conran and Dowd 1993, Chase *et al.* 1993), none of which occur in South Africa. The taxonomic and geographic isolation from its closest relatives suggest that *Roridula* is a paleoendemic genus (Linder *et al.* 1992). Paleoendemics are systematically isolated taxa with relatively ancient origins (Stebbins and Major 1965). They are often ecological specialists and are perhaps "on the way to extinction" (Stebbins and Major 1965). Their distribution range is relictual and their endemic condition is the result of a formally more extensive geographic distribution (Stebbins and Major 1965).

The only two species of hemipterans from the genus *Pameridea* (Miridae) are obligately associated with *Roridula* (one on each species, Dolling and Palmer 1991), suggesting that they are also paleoendemics and that the relationship between *Pameridea* and *Roridula* is a very old one. *Roridula* plants capture large amounts of insect prey using sticky traps although they have no digestive enzymes to digest this prey (Marloth 1910, Lloyd 1934, Ellis and Midgley 1996). They have a digestive mutualism with *Pameridea*, which feed on the captured prey (Ellis and Midgley 1996). They defecate on the leaves of *Roridula* and nitrogen is absorbed through the thin, non-continuous leaf cuticle of the plant (Ellis and Midgley 1996, chapter 4). *Roridula* plants can obtain up to 70 % of their total nitrogen from hemipteran faeces

(Anderson and Midgley, 2002). Thus the relationship between plants and bugs is species-specific and apparently obligate for both species.

In addition to *Pameridea*, Marloth (1903, 1910) also recorded a crab spider (*Synaema marlothi*) on populations near Tulbagh (Marloth 1903, 1910). In the mountains of the Cedarberg, Marloth (1925) found another large, green spider on *Roridula* that he did not identify. Dolling and Palmer (1991) also had a green spider from an unknown *Roridula dentata* locality identified as the lynx spider, *Peucetia* (Oxyopidae). Considering that *Peucetia* is the only large green spider routinely recorded on *Roridula*, the spider found by Marloth (1925) is most likely to be *Peucetia* as well. Marloth (1903, 1910, 1925) did not find the crab spider *Synaema* at the Cedarberg locality. The crab spiders have been shown to compete with *Pameridea* for nutrients and also consume the hemipterans (Anderson and Midgley, 2002). Populations with high spider densities generally have lower hemipteran densities and consequently these *Roridula* individuals are less efficient at obtaining nitrogen (Anderson and Midgley, 2002). By affecting hemipteran densities, it is thought that the indirect effects of spiders may strongly influence the reward quality for *Roridula* in the plant-bug mutualism. The evolution of obligacy and specificity between *Roridula* and *Pameridea* may have been strongly affected by the direct and indirect effects of other invertebrates on *Roridula*.

I aim to document the existing ranges of all fauna frequently associated with *Roridula*. From this, it is possible to determine how dependent associated fauna are on *Roridula*. Fauna with broader distributions than the distribution range of *Roridula* populations should be facultatively associated with *Roridula* because they are not entirely dependent on *Roridula* for their survival. However species with ranges that correspond exactly with the entire *Roridula* distribution (or part of *Roridula's* distribution) may be obligately associated with *Roridula* because this suggests that these species only occur in close association with *Roridula*. Secondly to determine whether present *Roridula* distribution patterns are due to vicariance events. One of Manter's (1955) rules of parasitism states that if the same, or two closely related species of host exhibit a disjunct distribution and possess similar, obligately associated parasite faunas, then the host distribution must have been contiguous at some time in the past. The same rule can be applied to *Roridula* because it has an association with a hemipteran genus that is obligately dependent on *Roridula* (see Dolling and Palmer 1991). Finally, using phylogeographic patterns, I aim to determine when relationships evolved between *Roridula* and its associated fauna.

METHODS

To document *Roridula's* general distribution, I used approximate localities (within 5km) acquired using information from the Bolus and Compton Herbarium. Exact localities were obtained from several sources including searches by the author, biologists, farmers and conservation authorities. Both exact and approximate localities were mapped in order to obtain a broad distribution pattern for the genus.

To document the invertebrate fauna, I directly observed plants from 23 populations over the course of a four-year period and over most of *Roridula's* range. By studying three populations (Pop4, Pop16 and Pop20, see Fig 1a) in different parts of *Roridula's* range, it was possible to observe all the invertebrate fauna with close associations with *Roridula* (i.e. the entire associated invertebrate fauna could be found in these three *Roridula* populations). Host specificity was indirectly determined in these three populations by "sweep netting" vegetation during five field trips over a four-year period. Taxa that are specific to *Roridula* should only be found on *Roridula* plants. Using a sweep net, I independently sampled *Roridula* plants and "non- *Roridula*" plants. In addition, I also used the sweep net to sample other glandular or hairy plants (especially *Elytropappus scaber*) within 1km of *Roridula* populations. The sweep net, was checked for invertebrates every minute, and the numbers of invertebrates captured (only those that commonly occur on *Roridula*) were noted.

RESULTS

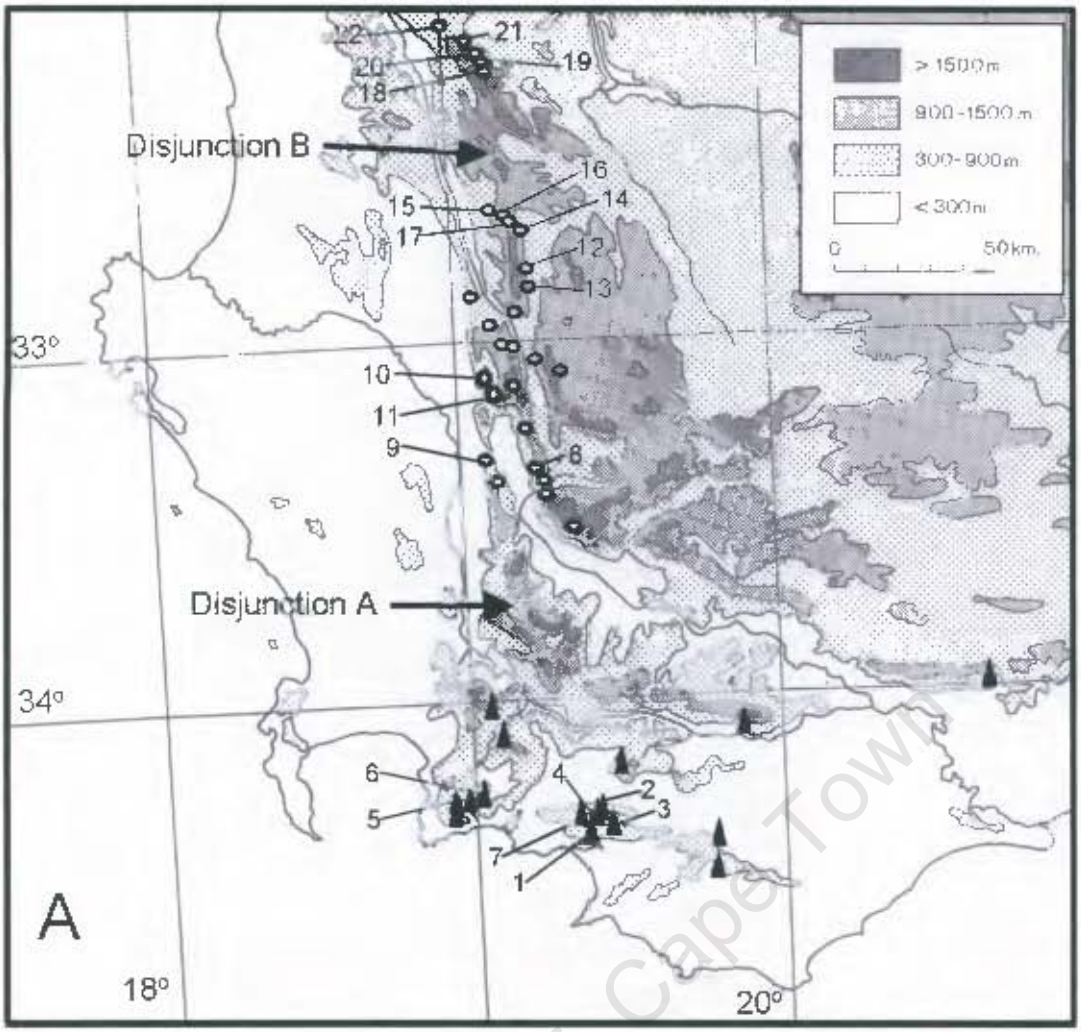
Roridula is geographically divided into two species with *Roridula gorgonias* occupying the southern mountains of the Riviersonderend, Perdeberge and Koggelberg and Langeberg (Fig. 1a). The two species are separated by approximately 70 km (disjunction A, Fig. 1a). *Roridula dentata* has a disjunct distribution starting in the Ceres region and following the Grootwinterhoek Mountains and Kouebokkeveld mountains to about Citrusdal. From Citrusdal to the Southern Cedarberg, there seem to be no records of *Roridula* populations (disjunction B, Fig. 1a). Searches in likely areas of the southern Cedarberg revealed no *Roridula* populations here. In addition, game guards and conservation authorities familiar with this area reported that they had never seen the plant in the southern Cedarberg, suggesting that the distribution data is correct. Several *Roridula* populations are found in the Northern Cedarberg (Fig. 1a) where game guards and conservation authorities knew the localities well. The plants of both species also tend to be found in isolated and very discrete populations. Populations can vary in size from between 5 and 2000 individuals. Usually plants are fairly densely packed, frequently touching

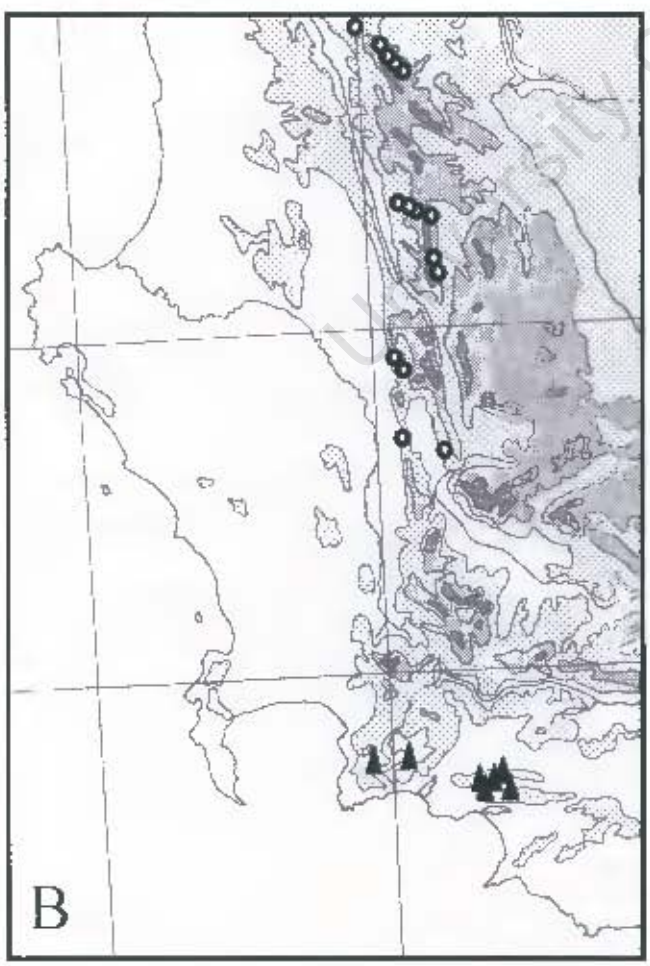
Figure 1.

1a. Map of *Roridula* distribution where labelled populations represent populations studied by the author, circles represent *R. dentata* populations and triangles represent *R. gorgonias* populations.

1b. Map of *Pameridea* distribution on populations studied by the author where circles represent the presence of *P. marlothi* on *Roridula* populations and triangles represent the presence of *P. roridulae* on *Roridula* populations. See Fig. 1a for contour key and scale.

1c. Map of *Peucetia nicolae* distribution on populations studied by the author where circles represent *Roridula* populations with *Peucetia* and triangles represent *Roridula* populations without *Peucetia*. See Fig. 1a for contour key and scale.





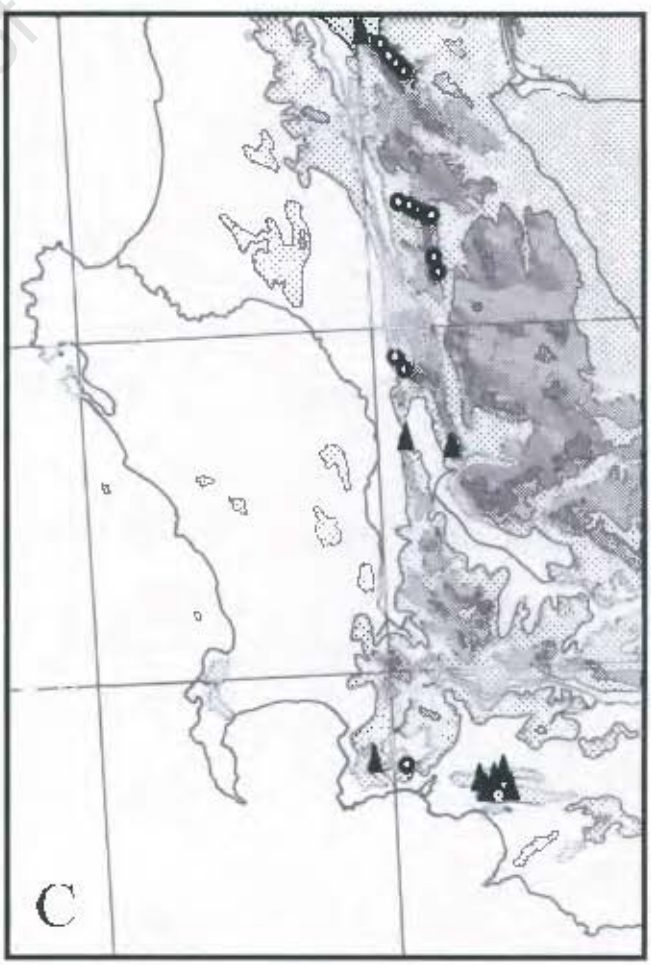
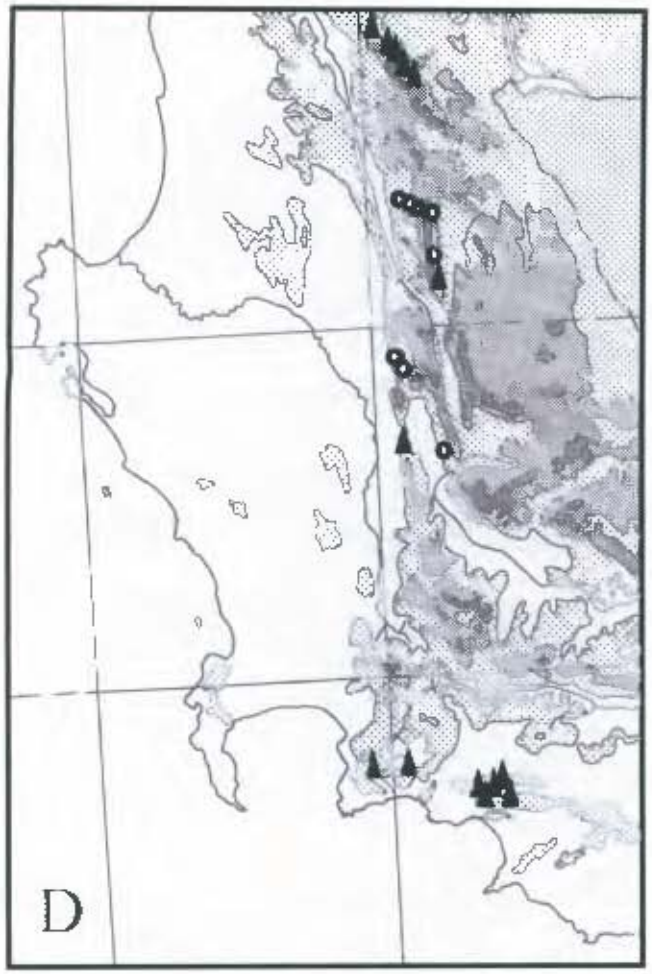


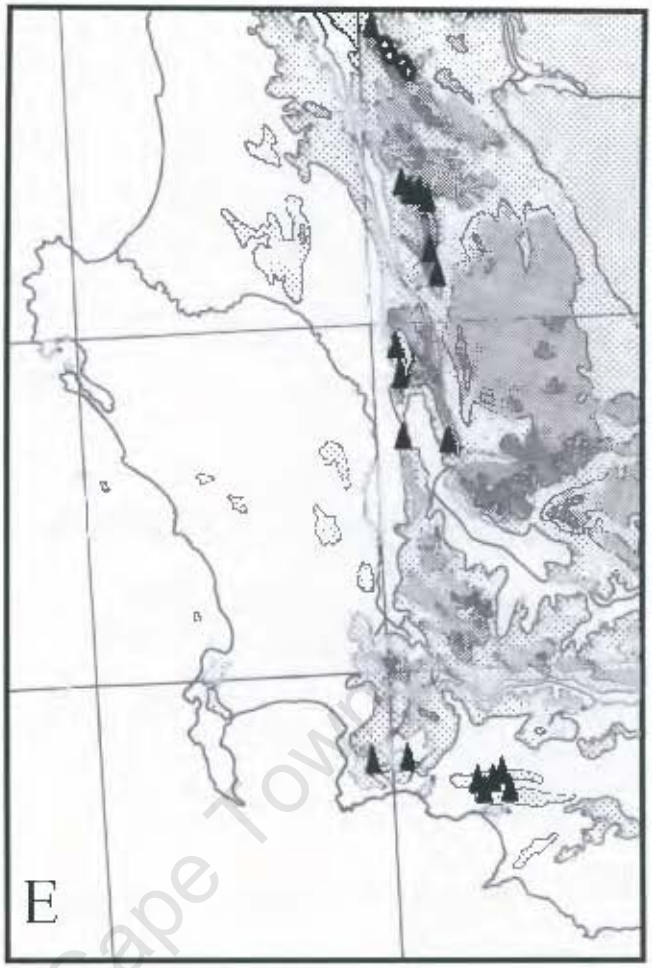
Figure1

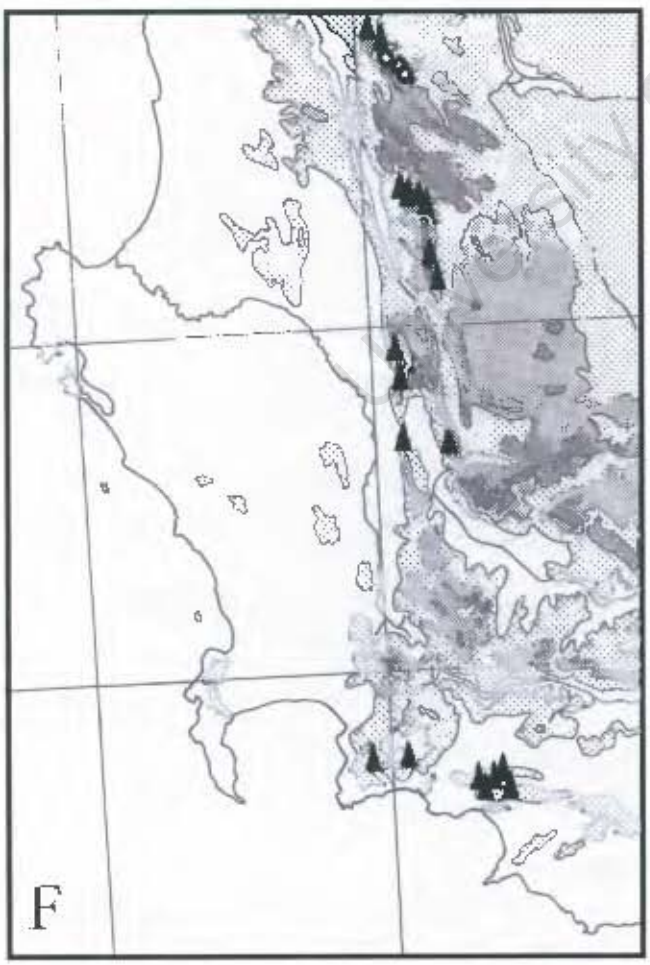
1d. Map of *Synaema* distribution on populations studied by the author where circles represent *Roridula* populations with *Synaema* and triangles represent *Roridula* populations without *Synaema*. See Fig. 1a for contour key and scale.

1e. Map of Araneid distribution on populations studied by the author where circles represent *Roridula* populations with Araneids and triangles represent *Roridula* populations without Araneid. See Fig. 1a for contour key and scale.

1f. Map of *Sphedanolestes sp*. distribution on populations studied by the author where circles represent *Roridula* populations with *Sphedanolestes* and triangles represent *Roridula* populations without *Sphedanolestes*. See Fig. 1a for contour key and scale.







one another. Old *Roridula* populations (> 10 years) are sometimes sparse and plants may be separated by up to 5 metres.

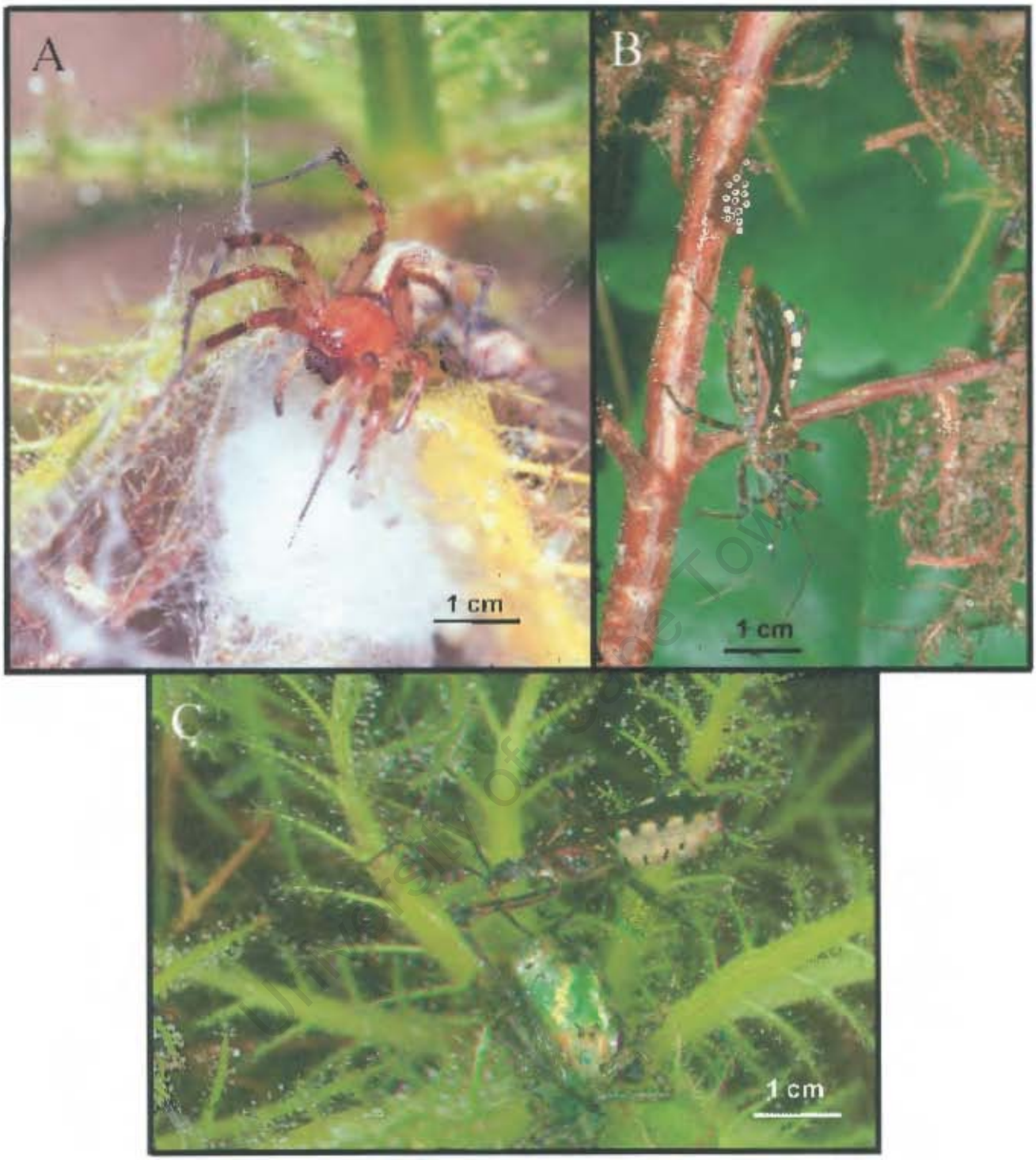
The distribution of *Pameridea* perfectly matches the distribution of *Roridula* (Fig. 1b). *Pameridea roridulae* was only found living is close association with *Roridula gorgonias* and *P. marlothi* with *R. gorgonias*. Neither species was recorded on any plants other than *Roridula* (Table 1). There were occasionally no *Pameridea* present on *Roridula* populations that were very recently burned (plants < 7 cm high). However, *Pameridea* were always present approximately one year after seedling germination. It is likely that *Pameridea* may take some time to recolonize *Roridula* populations after fire. Large plants sometimes had several hundred adult *Pameridea* on them and more than ten times as many juveniles.

Table 1. Catch per unit effort (SD) of *Pameridea*, an unidentified Araneid, *Synaema*, *Peucetia* and *Sphedanolestes sp.* on three different *Roridula* populations, random shrubs and viscid plants surrounding *Roridula* study sites. Included is the total time spent sampling in these habitats.

Population	Species	*Roridula*	Random	Viscid shrubs
Pop4	*Pameridea*	175 (53)	0(0)	0(0)
Pop4	Araneid	0(0)	0(0)	0(0)
Pop4	*Synaema*	0(0)	0(0)	0(0)
Pop4	*Peucetia*	0(0)	0(0)	0(0)
Pop4	*Sphedanolestes*	0(0)	0(0)	0(0)
Total time		10 min	120 min	0 min
Pop16	*Pameridea*	86 (33)	0(0)	0(0)
Pop16	Araneid	0(0)	0(0)	0(0)
Pop16	*Synaema*	6.9(3.3)	0(0)	0(0)
Pop16	*Peucetia*	2.4(1.7)	0.1(0.2)	3.6(2.8)
Pop16	*Sphedanolestes*	0(0)	0(0)	0(0)
Total time		10 min	150 min	150 min
Pop20	*Pameridea*	75 (32)	0(0)	0(0)
Pop20	Araneid	11(6.4)	0(0)	0(0)
Pop20	*Synaema*	0(0)	0(0)	0(0)
Pop20	*Peucetia*	3.4(2.8)	0.1(0.2)	3.7(3.0)
Pop20	*Sphedanolestes*	0.2(0.4)	0.01(0.1)	0.01(0.1)
Total time		10 min	180 min	180

I did not find any spiders associated with *R. gorgonias* during my first three years of study. However after the third year of observation, the lynx spider *Peucetia nicolae* (Dippenaar, pers. com.) appeared in the study site Pop6, after apparently being absent before (Fig. 1c). They reached densities of approximately one individual on every fifth plant. A year later, the spider was not to be found. This spider was not recorded in the southern *R. dentata* populations although it was recorded in the central populations of the Koue Bokkeveld and also the northern populations of the Cedarberg (Plate 6) where it sometimes reached densities of several individuals per

Plate 6A. An Araneid from the Cedarberg sits upon her nest. **B.** The reduvid, *Sphedanolestes sp.* lays her eggs upon the woody stem of *Roridula dentata* in the Cedarberg. **C.** *Sphedanolestes* consumes a large lynx spider (*Peucetia nicolae*), which it had killed earlier using a powerful venom injected by its proboscis.


A
1 cm
B
1 cm
C
1 cm

plant (Fig. 1c). *Peucetia* was absent from only a single population (Pop22) in the Cedarberg (Fig. 1c). This spider probably has a wide distribution and the type specimen was found at Grootvadersbos in the Langeberg with no mention of *Roridula* (Dippenaar, pers com.). I occasionally found *Peucetia* in sweeps on non-carnivorous plants (Table 1). When sweeping was confined to viscid and hairy plants (not *Roridula*) large numbers of *Peucetia nicolae* were captured (Table 1). They were especially common on the plant *Elytropappus scaber*. When these spiders were placed on *Roridula*, they were able to negotiate their way around the sticky traps.

The crab spider *Synaema marlothi* (Thomisidae) was found in large numbers on most of the populations in the southern and central range of *Roridula dentata* (Fig. 1d). *Synaema* was absent (Fig. 1d) from a single population in the South (Pop9) and from another in the central region (Pop13). The southern site Pop9 is very close to the area in which Marloth originally found the spider *Synaema marlothi*. The study sites Pop9 and Pop13 are small with only five plants in each. These plant populations would only be able to support very small spider populations and it is probable that spiders have gone locally extinct in both of these populations due to the negative genetic and/or demographic effects that influence small populations (e.g. Lande 1988, Saccheri *et al.* 1998). Sweep netting on non-carnivorous plants (including other hairy and glandular plants) yielded no *Synaema* specimens (Table 1). *Synaema marlothi* is abundant on the *Roridula* plants, often attaining densities of up to seven adults per plant. Nests are usually made in the axils of branches, close to the leaves. Spiders were observed to move quickly over the leaves to capture prey ensnared by *Roridula*. *Synaema* was also frequently observed consuming *Pamerídea*.

An additional spider (Plate 6) was found in the northern regions of the Cedarberg (Fig. 1e). The spider was identified by Dr. Ansie Dippenaar as belonging to the family Araneidae (orb-web spiders) and belongs to a genus previously unrecorded in South Africa. As the common family name suggests, Araneidae are known for their ability of constructing orb-webs. However, a few members within the family have reduced webs (Filmer 1991). The spider on *Roridula* has also lost the ability to construct an orb web and instead relies on the sticky plants to catch prey for them (pers. obs.). These are pounced on and consumed, negating the need to construct a web. Many orb weavers construct webs at night (Filmer 1991). However observations on this spider revealed no nocturnal web construction. Spiders do construct nests that are used as retreats and these are made on *Roridula* plants, in the axil of a leaf or small branch. The distribution of this spider is extremely geographically restricted and was found on 80% of the northern populations (Fig. 1e). It was absent from one northern population sampled (Pop22). Population size

is less likely to account for the lack of the Araneid in this population, as there are approximately 60 plants in the population PHP. However population Pop22 is very isolated and possibly represents the most northerly *Roridula* population known. On the populations where the Araneid did occur, it was fairly common, up to ten adults per plant. This Araneid was not found on plant species other than *Roridula dentata* (Table 1).

A species of hemipteran belonging to the family Reduviidae (*Sphedanolestes sp.*, Maxen, pers.com.) was also discovered (Plate 6) on 60 % of the northern populations of *R. dentata* (Fig. 1f). The reduvid was not found on any *Roridula dentata* populations from the central and southern regions (Fig. 1f). Nor was it found on *R. gorgonias* (Fig. 1f). The reduvid was found on two large populations of *Roridula* (Pop18 and Pop20) but always in very low densities. It was also found on a small population (Pop19) that was situated between the two large populations (Fig. 1f). It was observed laying its eggs on the stems of *Roridula* however the adults were only able to move very slowly over the sticky leaves where they hunted prey caught by *Roridula*. Other species of reduvid were frequently caught by *Roridula*. The reduvid was also found occasionally by sweep netting non-carnivorous plants (Table 1), suggesting that the relationship with *Roridula* is not obligate or species-specific.

Several other spiders were sporadically found on *Roridula dentata* including two species of jumping spider (Salticidae), another crab spider (*Synaema sp.* Thomsidae), a rain spider (*Palystes sp.*, Heteropodidae), a brown button spider (*Latrodectus geometricus*, Theriidae). None of these spiders were collected more than three times on *Roridula* plants and they are frequently found in other localities (pers. obs.). Their associations with *Roridula* appear extremely transitory and they are apparently facultatively associated with *Roridula*.

DISCUSSION

How dependent are associated fauna on Roridula*?*

Pameridea, *Synaema marlothi* and the unidentified Araneid are all found exclusively on *Roridula* plants suggesting that they are all host specific. This corroborates the suspicions of Dolling and Palmer (1991) that *Pameridea* is a host specific hemipteran. The lack of web construction in the Araneid suggests that this species has secondarily lost the ability to build webs because of its specialized life on *Roridula*. In contrast, Lynx spiders (*Peucetia*) were frequently captured while sweep netting viscous vegetation (other than *Roridula*). *Peucetia* is most likely an

opportunistic species with pre-adaptations that allow them to walk on *Roridula* plants (as well as other glandular plants) and exploit the rich resources. Similarly, some reduvids (*Sphedanolestes* sp.) were also captured on non-carnivorous plants suggesting that they too are only facultatively associated with *Roridula*.

Are present Roridula *distributions due to vicariance events?*

Roridula distributions are very disjunct with large distances (50-70 km) separating the different species and regions within a species. Nevertheless, *Pameridea* occurs on either side of these disjunctions. Disjunction "A" forms a species boundary, which can be used to define the two *Roridula* and *Pameridea* species. Thus, there is zero gene flow across this distribution gap. Chapter 8 also corroborates that there is no migration across this gene flow barrier. The most parsimonious explanation for this distribution pattern is that the common ancestor of *R. dentata* and *R. gorgonias* was once more widely distributed and hosted a single *Pameridea* species. Large-scale fragmentation possibly allowed allopatric speciation to occur simultaneously in *Roridula* and *Pameridea*. For a non vicariance model to have created these distribution patterns, both *Roridula* plants and *Pameridea* would have had to disperse across a gene flow barrier of (50-70km) in order to founder new populations. Since genetic data (Chapter 8) suggests zero gene flow between plant populations separated by more than a few kilometres, I suggest that it is unlikely that plant populations on either side of the disjunction could have been started through founder effects. Not only would plants have had to disperse widely, but *Pameridea* would also have had to disperse to and colonize the new *Roridula* populations at the same time. Since no migration occurs across disjunction A, I reject a non-vicariance model explaining the disjunct distribution patterns and propose that *Roridula* populations were once much more widespread.

Similarly, a large distribution gap (about 50 km) separates the northern *R. dentata* populations from the central/southern populations. The genetic structure of *P. marlothi* (chapter 8) suggests that the *Pameridea* on either side of this divide are incipient species and that gene flow between the northern and central populations is absent. Allopatric divergence, due to vicariance in *Roridula* distribution is the most parsimonious explanation for the observed genetic and geographic structure on either side of this disjunction. Judging from the taxonomic status (real species versus incipient species) of *Roridula* and *Pameridea* on either side of "disjunction A" and "disjunction B" respectively, it can be assumed that "disjunction A" occurred before "disjunction B".

Thus *Roridula* populations must have been more contiguous at one time. This is a common phenomenon in paleoendemic species, whose distributions have normally

resulted from vicariance events (Stebbins and Major 1965). *Roridula* is closely associated with moist habitats and the shrinking of these habitats may have caused the disjunct distributions observed for the genus. *Roridula gorgonias* occurs in seeps and marshy areas with permanent surface water (Carlquist 1976, pers. obs.), similar to the closely related Sarraceniaceae. *Roridula dentata* can be found on sandy "vlakte" (flats) that are drier than *R. gorgonias* localities in summer although underground water seems plentiful (Carlquist 1976, pers. obs.). In winter these areas are very wet (Carlquist 1976, pers. obs.).

How did fragmentation occur?

The fact that speciation has occurred across disjunction A for both *Roridula* and *Pameridea* suggests that vicariance occurred a long time ago and cannot be attributed to contemporary human disturbance. I suggest that some areas where *Roridula* once grew have become unsuitable for their growth due to climatic change. Until about 3.2-2.5 million years ago (late Pliocene), the Western Cape was characterized by a wetter summer rainfall (Deacon *et al.* 1992). Presently the Western Cape is typified by a winter rainfall and hot, dry Mediterranean type summers. Biome shifts associated with the change in climate may have constrained *Roridula* to high lying areas, which receive more rain. This may explain *Roridula's* affinity to relatively high lying areas. A similar theory has been invoked for the distribution of another rare paleoendemic, *Ixianthes retziodes* (Scrophulariaceae), which has a very similar distribution pattern to *Roridula dentata* (Steiner and Whitehead 1993, 1996). *Ixianthes* is also very closely associated with water and is only found on the banks of rivers.

Alternatively climatic oscillations in the last 2 million years could also have facilitated vicariance in *Roridula* and *Ixianthes*. In a recent climate change model Midgley *et al.* (2001) hypothesised that climatic oscillations may have shifted biomes along a North-South axis, which would also have forced taxa into topographic refugia. Presently the fynbos vegetation in which *Roridula* occurs is in a refugial condition, which may also account for the disjunct distributions and isolated populations. Vicariance events created by climatic change in either the Pliocene or Quaternary are likely to have been strong factors which promoted allopatric speciation in plant species (Midgley *et al.* 2001), especially those with low dispersal capabilities.

When did associations with Roridula *evolve?*

Three distinct distribution patterns can be observed in the associations between *Roridula* and their fauna. The first pattern is the perfect correlation between the distributions of *Pameridea* and *Roridula.* I postulated above (point 2) that *Pameridea* and *Roridula* speciated allopatrically after a single vicariance event. This supports the theory that *Roridula* and *Pameridea* were closely associated before the vicariance event took place. By similar reasoning, Pellmyr (1992) used distribution patterns of *Trollius* and their obligate fly pollinators (*Chiastocheta*) to infer evolutionary events in the two genera. He showed that a monophyletic group of five *Trollius* were associated with *Chiastocheta* throughout their ranges, suggesting that the common ancestor of that plant lineage had been associated with the fly lineage before it diversified. He rejected the possibility that the distribution patterns had arisen by secondary invasion throughout the present range of the plant. His reason was that this explanation requires repeated colonisations and invasions throughout the range of each species. It is therefore less parsimonious than the former hypothesis. I reject the secondary invasion hypothesis as an explanation of the *Roridula-Pameridea* distribution for the same reasons.

The second distribution pattern involves *Roridula* and two host specific spiders (unidentified Araneid and *Synaema marlothi*). Both of these species only occur on *R. dentata*, with each occupying only a small part of *R. dentata's* range. The *absence of these species from R. gorgonias suggests that associations between Roridula* and spiders only evolved after the vicariance event (disjunction A), which caused *Roridula* to speciate. Alternatively, these spiders could have had associations with *Roridula* before the vicariance event and then gone extinct in all the *R. gorgonias* populations. However, this explanation is not as parsimonious as the first as it requires repeated extinction events in all *R. gorgonias* populations (without *Pameridea* going extinct as well). Using this logic it is tempting to suggest that spiders only evolved associations with *Roridula* after the second vicariance event (disjunction B), as spider (*Synaema* and Araneid) distributions are constrained by this disjunction in *R. dentata's* distribution.

Finally, the distribution pattern of the lynx spider (*Peucetia*) is not constrained by either disjunction "A" or disjuncion "B" although it is not present in several of the *Roridula* populations. This distribution pattern reflects the non-host-specific nature of this species (i.e. disjunctions in *Roridula* distribution do not affect the movement or gene flow of *Synaema*). Thus vicariance of *Roridula* has not constrained the distribution of *Synaema* to certain regions, nor has it caused allopatric speciation of *Synaema* as in *Pameridea*. From the "haphazard" distribution of *Peucetia*, it is only

possible to say that this species is unlikely to be obligately associated with *Roridula*. It is difficult to determine when this loose association first occurred.

Conclusion

Distribution patterns suggest that *Pameridea* was closely associated with *Roridula* before ancient vicariance events caused speciation of the plants. Vicariance of plant populations and the low dispersal capabilities of *Pameridea* most likely caused cospeciation of the genera *Roridula* and *Pameridea*. Judging from their more limited distributions, it is likely that both the Araneid and *Synaema marlothi* have had shorter histories of close association with *Roridula* than *Pameridea* has had. Similarly, Pellmyr and Leebens-Mack (1999) suggest that cheating lineages of yucca moth are an order of magnitude younger than mutualist lineages. Marr *et al.* (2001) suggest that the age differences between these lineages may be because the conditions required for the evolution of cheating rarely occur. Alternatively, they suggest that cheaters commonly arise but only a small fraction persist. I concur with this and hypothesise that obligate mutualisms may need to be particularly well developed and stable in order to support exploitation by non-mutualists. It is probable that associations with exploiters have frequently caused the collapse of obligate mutualisms that have not had enough time (time without exploiters being present) to evolve a high degree of stability.

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PART 4

GENETIC DIVERSITY OF OBLIGATE MUTUALISTS

7 Genetic diversity of two rare mutualist genera (*Roridula* and *Pameridea*) and fitness in *Roridula*

ABSRACT

Habitat fragmentation may cause reductions in population size and the consequent genetic erosion of a single partner may have cascade effects on other mutualists. This is the first study to determine the effects of fragmentation on the genetic variability of two close mutualists, the sub-carnivorous plant *Roridula* and the hemipteran *Pameridea*. The allozyme diversity of *Roridula* was found to be low and is probably the result of genetic drift caused by historic and contemporary fragmentation. Despite low genetic variation, there was little evidence of inbreeding depression in *Roridula*. In contrast, the high genetic diversity in *Pameridea* apparently suggests good gene flow, indicating that *Pameridea* consists of metapopulations and that gene flow between populations maintains genetic diversity. The reduction in *Roridula* population size by ancient fragmentation events seems to have had little effect on the genetic variability of *Pameridea*. This highlights how the genetic diversity of organisms may be affected very differently by perceived fragmentation events (due to different dispersal capabilities and life history). However, the maintenance of all existing *Roridula* populations is crucial to the continued gene flow and high genetic diversity in *Pameridea* because *Roridula* populations probably serve as "stepping stones" for hemipteran movement. Finally, in order to conserve the remaining genetic diversity in *Roridula*, it is necessary to conserve a large number of populations because many populations have unique genetic combinations.

INTRODUCTION

The genetic consequences of habitat fragmentation (and consequent declines in population size) on species persistence have become a major source of concern to conservation biologists. Habitat fragmentation may shift the gene flow and resulting genetic structure of a species from panmixia (free gene flow throughout) or isolation by distance (attenuation of gene flow with distance) towards isolated, discrete, genetically distinct demes (Cunningham and Moritz 1998). Fragmentation is usually associated with a decrease in the effective population size (Selander 1983) and in small, isolated populations, genetic drift becomes the dominant influence on population genetic structure (Barrett and Kohn 1991). This reduces the genetic variation within populations and increases the variation among populations

(Selander 1983). Loss of genetic variability may reduce individual fitness and the ability of populations to respond to changing environmental conditions (Frankel *et al.* 1995, Lande 1995). The loss of variability over the entire species range may also make the entire species unable to adapt to large-scale change. This loss of "evolutionary potential" may not pose problems in the short term but may be threatening on an evolutionary time-scale (Keller and Waller 2002).

The other major effect of decreased population size is that drift may fix mildly deleterious alleles which increases the genetic load within a population (Keller and Waller 2002). However, the loss of fitness due to increased genetic load (termed "drift load") can only be observed by comparing the fitness of progeny resulting from crosses within the same population with that of progeny resulting from crosses between different populations (Keller and Waller 2002). Note that inbreeding depression (the decline in fitness observed in inbred progeny compared to outbred progeny) decreases as population size decreases (Bataillon and Kirkpatrick 2000, Keller and Waller 2002). The reason for this is that genetic drift in small populations removes genetic variability so that all individuals within a small population are genetically similar. As a result, selfed and crossed progeny will also be genetically similar and are liable to have the same fitness (Keller and Waller 2002). Hence very small populations should not exhibit inbreeding depression when the fitness of crossed and selfed progeny (from within the same population) are compared (Keller and Waller 2002). It is for this reason that the effects of genetic load can only be observed by comparing fitness of progeny crossed from the same population with that of progeny crossed from different populations.

In this study I am mostly concerned with the genetic diversity and the effects of genetic load in small populations. I examine the genetic diversity of a narrowly endemic plant family, Roridulaceae (consisting of a single genus - *Roridula*) and its very close mutualist, bug/hemipteran partner (*Pameridea*). *Roridula* is a paleoendemic, sub-carnivorous plant genus (see chapter 7), consisting of two geographically separated species (*R. dentata* and *R. gorgonias*), confined to a small region in South Africa (Obermeyer 1970). *Roridula* plants occur in very small (Tables 1a and 1b) and discrete populations. In addition *R. gorgonias* is a red data book species with a particularly narrow extant range. Chapter 8 examined the population genetic structure of both species of *Roridula* and found that they were very strongly subdivided at the population level and that there was little or no gene flow between even geographically close populations. This type of population structure is consistent with fragmentation and subsequent vicariance events. The high pair wise and hierarchical F_{st} values for *Roridula dentata* (chapter 8) also suggest a long history of isolation. Consequently, this genus is an ideal candidate for the study of long-term effects of fragmentation on genetic variability and fitness.

Roridula has a species-specific digestive mutualism with the hemipteran genus *Pameridea* (Ellis and Midgley 1996). These hemipterans are obligate mutualists and are only found on *Roridula* plants (Dolling and Palmer 1991). Two species are known: *Pameridea marlothi*, which only occurs on *Roridula dentata* and *Pameridea roridulae* that only occurs on *Roridula gorgonias* (Dolling and Palmer 1991). These two mutualists are thought to have been associated with each other before vicariance events fragmented plant populations and the mutualism is probably ancient (see chapter 7). *Pameridea* is winged and may be a better disperser than *Roridula*, which has no obvious seed dispersal adaptations (pers. obs.). This possibly allows *Pameridea* to move between close populations whereas gene flow between populations of *Roridula* plants is unlikely (chapter 1). *Pameridea* populations are also larger than *Roridula* populations because each *Roridula* plant can support several adult *Pameridea* (Dolling and Palmer 1991). As a consequence of their better dispersal abilities and larger population sizes, genetic subdivision is not as marked in *Pameridea* as it is in *Roridula* (chapter 8). On a regional scale, hemipteran F_{st} values were low (< 0.03) indicating fairly good gene flow between populations (although some close populations had significantly different genetic structures). It is likely that *Pameridea* is a metapopulation where the species range is composed of geographically isolated patches, interconnected by patterns of gene flow, extinction and recolonization (Levins 1970). Comparing the genetic diversities of these two taxa with such different dispersal characteristics and life histories is interesting considering that they share exactly the same distribution range.

Juvenile *Pameridea* are also the primary pollinators of *Roridula* flowers and since they are flightless, the majority of seed set by *Roridula* is probably selfed (chapter 1). Although *Roridula* flowers have features that suggest bee pollination (Vogel 1978, Johnson 1992), bee pollination is very rare (chapter 1). In order to set adequate quantities of seed, *Roridula* rely on *Pameridea* for facilitated selfing (chapter 1). However, selfing is only advantageous if selfed seeds are viable, germinate and reach maturity (Lloyd 1979). If inbreeding depression is high, facilitated selfing by hemipterans may be detrimental to *Roridula* and not beneficial as results from chapter 1 suggest.

I examine the genetic diversity in *Roridula* to identify what effects fragmentation and selfing may have had on it. In particular, I ask whether diversity in *Roridula* populations is low and therefore in concordance with a post fragmentation scenario. I also investigate genetic diversity in *Pameridea* to determine whether fragmentation of *Roridula* populations has in turn caused fragmentation and loss of genetic diversity in *Pameridea* populations. The comparisons of genetic diversity in *Pameridea* and *Roridula* are important because organisms with close, obligate

mutualisms may be threatened by the extinction of their partners (although not always immediately, Bond 1994). Hence the genetic diversity of one mutualist partner may have long-term effects on the other partner as well as itself. To my knowledge, this is the first study to examine genetic diversity in two mutualist partners from a conservation perspective. I also correlate various measures of genetic diversity with population size in *Roridula* and *Pameridea* and I also correlate genetic diversity measures of *Pameridea* with those of *Roridula*. Seed set, seed germination and seedling mortality are also examined from two *Roridula* populations to infer levels the effects of genetic load on plant fitness. I hypothesize that very high seed set, high seedling germination and very low seedling mortality would suggest that the adverse effects of inbreeding depression are minimal without having to compare the fitness of crossed progeny within and among populations. This would represent a reasonable, first approximation of the effects of fragmentation on fitness, however, it is not a real measure of inbreeding depression.

METHODS

Genetic variability

For study sites, collection and electrophoresis methods, see chapter 8. The number of alleles per locus and the percentage of polymorphic loci are sensitive indicators of bottlenecks and small population size (Nei *et al.* 1975, Leberg 1992) because bottlenecks affect these measures immediately after reductions in population size. Several studies support the theory that polymorphism and allelic diversity are reliable indicators of previous bottlenecks (e.g. Briscoe *et al.* 1992, McCommas and Bryant 1990, O'Brien *et al.* 1983, Leberg 1992, Thomas and Bond 1997). However heterozygosity is normally only affected if a population takes a long time to recover from a bottleneck (Leberg 1992). Hence I examined both locus polymorphism and levels of heterozygosity to infer the effects of habitat fragmentation on genetic diversity. The genetic diversity was calculated for each population by Nei's (1978) unbiased estimate of expected heterozygosity (H_e), observed heterozygosity (H_o), percentage polymorphic loci (P), and the mean number of alleles per locus. A locus was considered polymorphic if it had more than one allele. These measures were calculated using the computer program BIOSYS-1 (Swofford and Selander 1981). The number of plants and hemipterans were estimated in each population and these were correlated with the estimates of genetic diversity. In addition, estimates of hemipteran genetic diversity were correlated against estimates of plant genetic diversity.

Plant fitness

The effects of inbreeding depression are preliminarily examined by investigating plant fitness during the early stages of *Roridula's* life history. If inbreeding depression is calculated in small populations by comparing the fitness of crossed and selfed progeny, a lack of evidence of inbreeding depression may arise because a large proportion of genetic load is likely to be fixed (i.e. crossed progeny are fixed for the same alleles as selfed progeny because both reflect the effects of population inbreeding). Instead, Keller and Waller (2002) suggest that the fitness of progeny crossed within a population should be compared with that of crosses from among populations. However, I argue that if seedset and germination rates are very high, and seedling mortality is very low, then among population crossed are unlikely to increase the fitness of progeny. Thus, high seed set, high germination rates and low seedling mortality may suggest that the effects of fragmentation are minimal in terms of plant fitness. Note that this is only a first approximation of fitness and not a measure of inbreeding depression as outlined by Keller and Waller (2002). Thus I examine seed set, germination rates and seedling mortality within populations to determine whether they are close to optimal levels.

Sixteen *R. gorgonias* flowers (one from each plant) and 30 *R. dentata* flowers were hand crossed. Self-pollination by hand was also carried out on the same numbers of *R. gorgonias* (Population 1) and *R. dentata* flowers (Population 8). After pollination, flowers were emasculated and a nylon bag was placed around each flower to prevent cross-pollination. Several months later, the capsules were examined for seed set and the numbers of seeds per capsule were counted. To calculate percentage seed set, I assumed that *R. gorgonias* has a mean of nine ovules per capsule (Obermeyer, 1970 estimates that *R. gorgonias* has three locules and two-four ovules per locule). I also assume that *R. dentata* has an average of three ovules per capsule (Obermeyer 1970). Three seeds from each capsule were chosen (one per locule), subjected to smoke treatment (seeds germinate after fire) and allowed to germinate in direct sunlight in a medium of moist tissue paper.

During the course of this study, two populations burned down, providing an opportunity to study natural seedling recruitment, mortality and bottlenecks of hemipteran populations. One was a *R. gorgonias* population (Pop5, estimated 250 plants and 6250 adult hemipterans). This population burned in December 1999, leaving no surviving *Roridula* or *Pameridea*. *Roridula* seedlings germinated approximately six months later and hemipterans were found in very low densities approximately one year later (February 2001). Hemipterans must have colonized from another population and thus undergone a founder effect. I monitored seedling mortality and the increase of hemipteran numbers over the period of one year. The other population was a *R. dentata* population (Pop8), which burned in November 1999 leaving 6 surviving plants and 60 surviving hemipterans. I document the

seedling mortality in this population and the recovery in hemipteran numbers between the period of February 2001 and February 2002. Approximately one year after the fires at both of these sites (February 2001), permanent one metre by one metre quadrats were placed within the *Roridula* populations. At Pop8, four quadrats were placed randomly as there was no area where seedling recruitment was obviously better than others. However, in Pop5, water availability was steeply clined and seedlings recruited preferentially in wet sites. Here, quadrats were placed across the cline and spanned peripheral areas where seedling recruitment was poor and core areas where seedling recruitment was good. Two quadrats (one by one metre) were placed in peripheral areas (less than 11 seedlings per m^2) and two quadrats were placed in core areas (more than 79 seedlings per m^2). The number of seedlings were counted and then recounted in each quadrat one year later.

RESULTS

Genetic variation (Tables 1a-d)

In *Roridula gorgonias*, only a single polymorphic locus (1/9) was found. Polymorphism was found in two populations while the remaining four populations were monomorphic for all alleles (Table 1a). The average number alleles per locus (over all populations) was estimated as 1.03 and the average observed and expected heterozygosities were low with no value larger than 0.06 (Hamrick and Godt, 1996 reported $H_e = 0.063$ for endemic plant species). I assume that Hamrick and Godt (1996) define endemic species as taxa, which have very narrow distributional ranges. Expected heterozygosity was an order of magnitude greater than observed heterozygosity and the difference between the two was significant in each population ($p < 0.05$).

Table 1a. Genetic variability in *R. gorgonias* where N = number of individuals in the population, P = percentage of polymorphic loci, A = mean number of alleles per locus, H_e = expected heterozygosity (SE), H_o = observed heterozygosity (SE)

Population	N	P	A	H_e	H_o
Pop1	50	0.0	1.00	0.00	0.00
Pop7	700	0.0	1.00	0.00	0.00
Pop3	40	11.1	1.11	0.013 (0.013)	0.00
Pop2	1200	11.1	1.11	0.057 (0.057)	0.005 (0.005)
Pop6	150	0.0	0.00	0.00	0.00
Pop4	70	0.0	0.00	0.00	0.00
Mean		3.6	1.03	0.012	0.001
Total over all pops.		11.1	1.11		

In contrast, 58.3 % of all loci examined for *R. dentata* were polymorphic (Table 1b) although very little of this variation was found within populations. The population with the most polymorphic loci was also the smallest population with 25 % locus polymorphism. Mean locus polymorphism was 6 % averaged over all populations. Most locus polymorphism in this species represents fixed allele differences between populations and thus the number of alleles per locus in each population was low (1.00 - 1.25) where values of 1.00 represent total fixation at all loci. The average number of alleles per locus (in each population) was very low (1.06) whereas the total number of alleles per locus was much higher (1.67). The average heterozygosity was also very low with values of both H_o and H_e being lower than 0.06 in all populations. Expected heterozygosity was an order of magnitude greater than observed heterozygosity and the difference was significant in each population ($p < 0.05$).

Table 1b. Genetic variability of *R. dentata*.

Population	N	P	A	H_e	H_o
Pop8	1000	0.0	1.00	0.000	0.000
Pop15	100	8.3	1.08	0.015 (0.015)	0.000
Pop16	90	8.3	1.08	0.037 (0.037)	0.000
Pop17	30	0.0	1.00	0.000	0.000
Pop12	45	0.0	1.00	0.000	0.000
Pop14	6	0.0	1.00	0.000	0.000
Pop22	80	8.3	1.08	0.009 (0.009)	0.000
Pop19	5	25.0	1.25	0.049 (0.029)	0.008 (0.008)
Pop18	350	8.3	1.08	0.018 (0.018)	0.000
Pop20	400	8.3	1.08	0.01 (0.01)	0.000
Pop21	6	0.0	1.00	0.000	0.000
Mean		6.0	1.06	0.013	0.001
Total over all pops.		58.3	1.67		

In *P. marlothi*, 43.8 % of all loci examined were polymorphic (Table 1c) and a substantial part of this polymorphism was found within individual populations. In the majority of populations, over 30 % of all loci were polymorphic and in one population (Pop1), locus polymorphism equalled that found over the entire species. Very low

locus polymorphism was found in a single population (VRD) that also had low numbers of plants (n = 5) and hemipterans (n = 50). The numbers of alleles per locus was higher than that found in any of the *Roridula* populations and high numbers of alleles per locus were found in individual populations. Expected and observed heterozygosities were low (mean = 0.073 and 0.064 respectively) but nevertheless larger than those found in *R. gorgonias*. Expected and observed heterozygosities did not differ significantly in any population ($p > 0.05$).

Table 1c. Genetic variability of *P. roridulae*.

Population	N	P	A	H_e	H_o
Pop1	1200	43.8	1.63	0.095 (0.044)	0.086 (0.038)
Pop7	1750	31.3	1.44	0.067 (0.038)	0.050 (0.028)
Pop3	100	6.3	1.13	0.039 (0.039)	0.033 (0.033)
Pop2	960	37.5	1.69	0.083 (0.044)	0.075 (0.037)
Pop6	660	37.5	1.63	0.080 (0.041)	0.074 (0.036)
Mean		31.3	1.50	0.073	0.064
Total over all pops.		43.8	1.88		

In *P. roridulae*, 66.6 % of all loci examined were polymorphic (Table 1d) and a substantial part of this polymorphism was found within individual populations. Every measure of genetic diversity suggested greater diversity in *P. marlothi* than in *P. roridulae*. In all of the *P. marlothi* populations, over 30 % of all loci were polymorphic and some of these populations were small (< 100 individuals). The numbers of alleles per locus (at all hierarchical levels) was higher in *P. marlothi* than in all other species analysed in this study. Expected and observed heterozygosities were high (mean = 0.112 and 0.104 respectively) and did not differ significantly in any population ($p > 0.05$).

Table 1d. Genetic variability of *P. marlothi*.

Population	pop size	P	A	H_e	H_o
Pop8	7000	53.3	1.60	0.089 (0.031)	0.080 (0.030)
Pop12	495	60.0	1.93	0.140 (0.048)	0.131 (0.042)
Pop13	50	33.3	1.53	0.093 (0.045)	0.090 (0.043)
Pop15	2000	53.3	1.80	0.143 (0.052)	0.127 (0.043)
Pop16	1620	40.0	1.53	0.102 (0.044)	0.104 (0.043)
Pop14	200	33.3	1.40	0.112 (0.051)	0.103 (0.046)
Pop22	1200	33.3	1.47	0.112 (0.053)	0.111 (0.054)
Pop18	3000	53.3	1.67	0.118 (0.040)	0.100 (0.036)
Pop20	4800	46.6	1.67	0.100 (0.041)	0.094 (0.040)
Mean		48.9	1.62	0.112	0.104
Total over all pops.		66.6	2.27		

The Pearson product-moment correlation coefficient suggested no relationship between any of the genetic diversity measures and population size of any species

analysed ($r < 0.6$, $p > 0.1$ for all correlations). Nor were any of the genetic diversity measures of hemipterans correlated with those of the plants on which they live ($r < 0.3$, $p > 0.5$ for all correlations).

Plant fitness

Fruit set in *Roridula gorgonias* was very high (in excess of 80%) irrespective of whether the seeds were produced by cross or self-pollination (Fig. 1a). Similarly, fruit set of self and cross-pollinated flowers was in excess of 95% for *Roridula dentata* (Fig. 1b). The percentage seed set for both crossed and selfed *R. gorgonias* flowers was high (selfed seeds = 100 % and crossed seeds = 89 %. Fig 1a) and that of *R. dentata* was in excess of 90% (Fig 1b). The difference between the numbers of crossed and selfed progeny was not statistically significant for fruit set or seed set in *R. dentata* and *R. gorgonias* (Fisher's Exact Test, $p > 0.2$). Nor was seed germination significantly different for selfed or crossed seeds. In *R. gorgonias*, 80 % (36/45) of crossed seeds germinated and 73 % (33/45) of selfed seeds germinated ($\chi^2 = 0.07$, $p > 0.7$, Figure 1a).

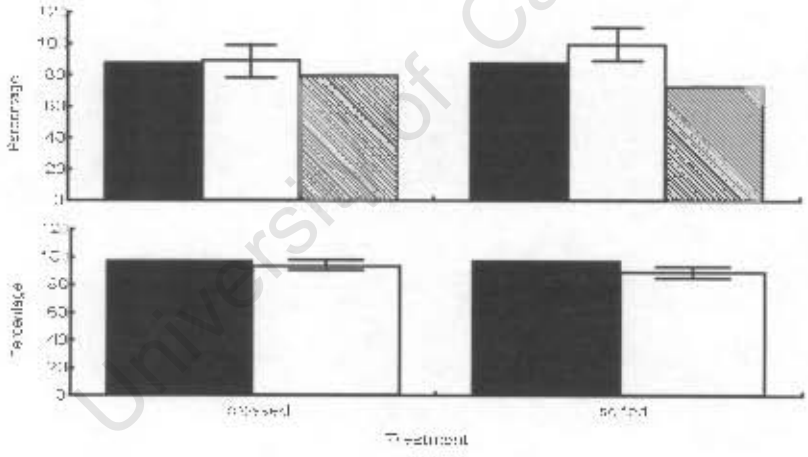

Figure 1a. Percentage fruit set (shaded column), seed germination (hatched column) and mean seed set (clear column) for *R. gorgonias*.
Figure 1b. Percentage fruit set seed set for *R. dentata*.

In *R. gorgonias*, a total of 12.7 % of plants died during the first year of growth (Figure 2a). Much of this can be attributed to self-thinning as seedlings were densely packed (about 100 m^{-2} in moist areas). While seedling numbers changed very little over the course of the year, the mean plant height increased from 10.4cm to 32.8 cm ($Z = -16.86$, $p < 0.001$, Mann-Whitney U). The average number of adult hemipterans per plant also increased from approximately 0.02 to 0.99 ($Z = -12.71$, p

< 0.001, Mann-Whitney U). This represented a total adult population increase from about zero in 1999 to 196 in 2001 and finally 8537 in 2002. For *R. dentata*, there were few seedlings per plot and therefore probably little competition. I hypothesize that low plant density contributed substantially to the low plant mortality (4%) within the first year. During this time, plant height increased significantly from 8.0 cm to 23.3 cm ($Z = -5.78$, $p < 0.0001$, Mann-Whitney U). Hemipteran density also increased from 0.08 hemipterans per plant to 2.25 per plant ($Z = -5.23$, $p < 0.0001$, Mann-Whitney U). The total hemipteran population size increased from about 60 adult individuals in December 1999 to 240 in February 2001 and finally to 6480 individuals in February 2002.

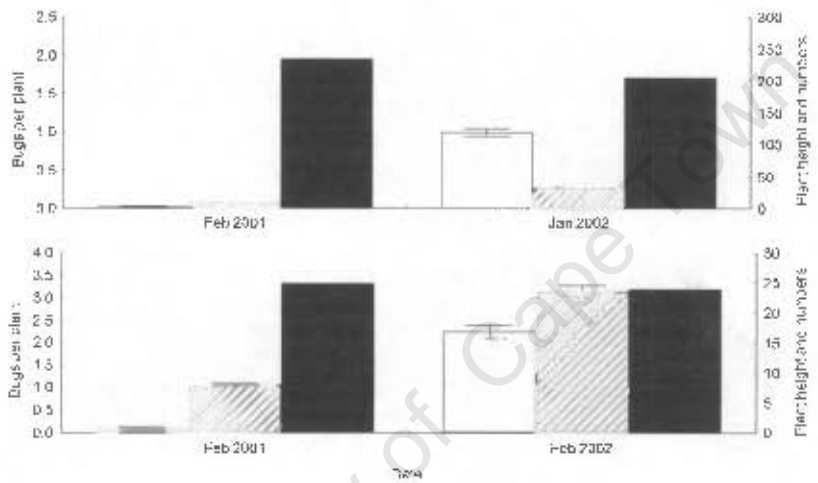

Figure 2a. Average number of hemipterans per plant (clear column), mean plant height (hatched column) and total number of plants in six quadrats (shaded column) in a *R. gorgonias* population (Pop5).
Figure 2b. Average number of hemipterans per plant, mean plant height and total number of plants in four quadrats (shaded column) in a *R. dentata* population (Pop8).

DISCUSSION

Roridula is a narrowly endemic (Obermeyer 1970), self-pollinating species (See chapter 1) and this paper suggests that the genus has very low genetic variability at the population level although there are strong genotypic differences between populations. In addition, there is no preliminary evidence of a strong loss in fitness as a result of inbreeding depression acting on *Roridula* populations. However, inter-population crosses should be made to confirm these results. In contrast, the hemipteran *Pameridea* exhibits much higher genetic variability at both the population and species level. The low genetic variability of *Roridula* is consistent with other narrowly endemic and selfing plant species which have been shown to be

genetically depauperate Hamrick and Godt (1990). In fact some have little or no variation at the loci examined (Ledig and Conkle 1983; Waller *et al.* 1987). But on average, 41.8% of a selfing plant species' loci are polymorphic (at the species level) and 20.0 % of all loci are polymorphic within a population (Hamrick and Godt 1990). Fragmented species with small populations are also likely to have very low genetic diversity within populations. In a South African example, Matolweni *et al.* (2000) found a mean of 5.7 % polymorphic loci per population for *Begonia homonyma*. Mean numbers of alleles per locus and observed heterozygosity were also low (1.11 and 0.027) respectively. However, *B. homonyma* occupies a fairly large range (> 200km) and as a result genetic diversity for the entire species was high (73 % polymorphic loci and 2.00 alleles per locus). *Roridula gorgonias* is a very narrow endemic with small populations and consequently locus polymorphism is low at both the species level and the population level. The percentage of polymorphic loci within and over all populations in *R. gorgonias* (3.6 % and 11.1 % respectively) is well below the average of most selfing species (20.0 % and 41.8 % respectively, Hamrick and Godt 1990) reflecting the effects of drift and high selfing rates suggested in chapter 1, small population sizes and narrow distribution. Although number of alleles per locus and heterozygosity were also low in *R. gorgonias*, these values should be treated with caution as only one polymorphic locus was scored and these values may not be representative of allelic diversity or heterozygosity within the species. Similar to Ledig and Conkle (1983), I believe that bottlenecks caused by a change from summer to winter rainfall (approximately 3.5-2.5 myr ago) may have fragmented populations leading to reduced genetic diversity in *R. gorgonias*. Steiner (1993, 1996) also used climate change during this time to explain the disjunct distribution pattern of another rare paleoendemic (*Ixianthes retziodes*). The effects of continued inbreeding would have exacerbated the fragmentation effects and further decreased genetic diversity.

For *Roridula dentata*, all measures of genetic diversity within populations were comparable to those found by Matolweni *et al.* (2000) and they were much lower than the values of other selfing species and narrow endemics (Hamrick and Godt 1990). The range of *Roridula dentata* is of similar extent to *Begonia homonyma* and as a result, the genetic diversity over all populations is much greater than *R. gorgonias* and comparable to *Begonia*. Heterozygote deficiencies in *R. dentata* are an inevitable consequence of selfing and mating with closely related individuals due to small population size. I hypothesize that similar events may have caused fragmentation in these two closely related species and since the pollination biology of *R. dentata* and *R. gorgonias* is also similar, the genetic variation within populations of both species are very low. The very low genetic diversity of both *Roridula* species suggests severe bottlenecks in the past.

Waller 2002). Although no crosses were made between populations, such crosses are unlikely to significantly improve on the rates of within population fruit set, seed set, germination or seedling mortality observed in this study. The effects of genetic load seem absent in the early life stages, although it may be significant in the later stages of the plants' life history due to the expression of mildly recessive alleles (Husband and Schemske 1996). Chapter 1 suggests that the selfing rates in *Roridula* populations are high and chapter 7 indicates that germination rates of selfed seeds are also very high (relative to crossed seed). Thus I assume that the majority of seedlings in any population are selfed progeny. The results in this chapter (chapter 7) also suggest very low seedling mortality in *R. dentata* and *R. gorgonias*, which indicate that selfed progeny are likely to reproduce (*Roridula* plants are able to set seed approximately two to three years after germination). Thus self-pollination by hemipterans in the absence of bee pollinators probably does play an important reproductive assurance role in *Roridula*. I can therefore extend the mutualism from a digestive mutualism to include pollination as well.

Although the adverse affects of selfing, inbreeding and genetic drift are not evident as a decrease in the fitness of *Roridula* (e.g. low seed set, low rates of seed germination, high seedling mortality), they undoubtedly have strong influences on the genetic diversity within populations. Even though inbreeding depression may be negligible, the inability to adapt (due to low genetic diversity) to changing biotic and biotic factors may prove to be detrimental in the long term (Franklin 1980). Low genetic diversity may also constrain the ability of a species to colonize new areas. However the study of Red Pine (*Pinus resinosa*) indicates that low genetic variability may not affect the ability to colonize aggressively (Millar and Libby 1991). Although the fragmentation of *Roridula* populations has not affected the genetic diversity of hemipterans, the presence of many close *Roridula* populations plays a vital role as "stepping stones" for gene flow and recolonization of *Pameridea*. This minimizes the loss of genetic diversity and decreases the probability of permanent, local extinction events in *Pameridea*. Because the long-term survival of the mutualism depends on the fitness of both *Roridula* and *Pameridea*, conservation efforts cannot act independently on either *Roridula* or *Pameridea*. In order to conserve the genetic variability of *Roridula*, it is necessary to conserve populations from many different regions because different regions frequently have unique combinations of genetic characters (especially in *R. dentata*). However it may be even more important to maintain numerous close *Roridula* populations within a region so that gene flow corridors and the metapopulation structure of *Pameridea* persist (i.e. maintenance of connectivity). Lawton *et al.* (1994) suggest that metapopulations are able to persist until a certain threshold number of habitat patches are destroyed - after this the metapopulation will collapse. Unfortunately this threshold number of populations will differ from species to species due to differences in dispersal and life

history, thus it is impossible to predict how much fragmentation is required to disrupt the *Roridula-Pameridea* mutualism.

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PART 5

SPECIATION AND LOCAL ADAPTATION

8 Comparative population genetic structures of *Roridula* and *Pameridea*: cospeciation through vicariance

ABSTRACT

Similar patterns of dispersal and gene flow between closely associated organisms may promote local adaptation and coevolutionary processes. We compare the genetic structures of a plant genus (*Roridula gorgonias* and *Roridula dentata*) and its obligately associated hemipteran mutualists (*Pameridea roridulae* and *Pameridea marlothi*) using allozymes. Allozyme variation was found to be very structured among plant populations but less so among hemipteran populations. Strong genetic structuring among hemipteran populations was only evident when the plant populations on which they live were isolated by large distances. Although genetic distances among plant populations were correlated with genetic distances among hemipteran populations, genetic distances of both plants and hemiptrerans were better correlated with geographic distance. Because *Roridula* and *Pameridea* have different scales of gene flow, adaptation at the population level is unlikely. However, the restricted gene flow of both plants and hemipterans could enable adaptation to occur at a regional level.

INTRODUCTION

The level of local adaptation in any species depends on the interaction between gene flow and the strength of selection, as well as demographic parameters (review by Lenormand 2002). Evidence for adaptation to host phenotype has occasionally been inferred by showing that the genetic structures for so called neutral traits (e.g. allozymes) are correlated for hosts and their parasites (Mulvey *et al.* 1991, Demasters and Hafner 1993). However, correlated genetic structures may also be caused by hosts and their parasites having similar dispersal/distribution patterns. As a result, genetic studies on local adaptation should be combined with experimental studies. Correlated genetic structures resulting from similar dispersal/distribution patterns may also enhance the potential for local coadaptation to occur.

Many monophagous insects are poor dispersers and form demes which are genetically distinct groups of the same species, in close proximity (Mopper 1996). Genetic differences are particularly evident when host plants are patchy or insect

gene flow is low (e.g. Rank 1992, Alstad and Corbin 1990). Adaptive deme formation is thought to occur if host plants are phenotypically different (Strauss 1990, Boecklen *et al.* 1990, Auerbach 1991, Mopper and Simerloff 1995) and strong selection pressures of the different plant phenotypes act as disruptive selection mechanisms (Peterson and Denno 1998). In addition to selection, dispersal ability also plays an important role in adaptive deme formation because very strong gene flow may swamp local adaptation despite strong local selective pressures (e.g. Raymond and Marquine 1994, Storfer 1999). In contrast, very low gene flow may preclude local adaptation by limiting genetic variability (Gandon *et al.* 1996).

It is therefore crucial to document the extent of gene flow among populations of a given species, so that predictions about local adaptation can be made. Most studies make use of neutral markers to infer gene flow, although gene flow might be directly inferred from population structure of genes involved in an interaction. An example of this is the study by Lenormand *et al.* (1999) on the use of genes for insecticide resistance to measure gene flow. Predictions about the outcome of heterogeneous selection can then be tested using the traits involved in the local adaptation. Although the use of F_{st} has been questioned several times (e.g. Bossart 1998), recent studies have shown that F_{st} seems a good surrogate to measure dispersal ability of a species (Bohonak 1999, Neigel 2002).

In the context of interacting species, it has been suggested that the difference between host and parasite migration rates may explain the level of local adaptation of both species (Gandon *et al.* 1996, Gandon 2002). These studies suggest that for low to intermediate migration rates, parasites should become locally adapted if they migrate more than their hosts and *vice versa*. However if migration rates are high or similar for both species, then there should be no local adaptation. Such predictions were successfully tested (Kaltz *et al.* 1999). Although it is not known how these predictions might apply to mutualistic systems, it is likely that the amount of gene flow also influences coevolutionary outcomes in such systems (Thompson 1994, Gomulkiewicz *et al.* 2000).

Coevolutionary outcomes may be strongly affected by the geographical distribution patterns of interacting organisms and their relative abilities to traverse gene flow barriers. Thus, comparative genetic structures of closely associated organisms are useful to evaluate the geographical scale at which coevolutionary interactions take place (Althoff and Thompson 1999). Distance and various barriers might constrain the dispersal in each species and this might lead to strong correlation between spatial structures. In mutualistic systems, this might facilitate coevolution and possibly cospeciation. However, interacting organisms often have incongruent patterns of genetic structure (e.g. Althoff and Thompson 1999) and this could

influence the scale at which local adaptation is likely to occur. Although some recent studies have compared population structure of interacting species (Mutikainen and Koskela 2002, Delmotte at al. 1999, Martinez *et al.* 1999, Dybdahl and Lively 1996, Mulvey *et al.* 1991, Jerome and Ford 2002a, 2002b, Michalakis *et al.* 1994), no study has considered mutualistic organisms, with the exception of Parker and Spoerke (1998) on *Amphicarpa* and *Rhizobium*. A geographical perspective as used by Althoff and Thompson (1999) is crucial in understanding these interactions better.

In the context of plant-insect interactions, several studies have examined the genetic structure of either parasites or hosts. For example, sympatric host races of the apple maggot fly (*Rhagoletis pomonella*) have been identified using allozyme electrophoresis (Feder *et al.* 1988; Feder *et al.* 1990, McPheron *et al.* 1988). However, in both cases, host plants belonged to different species and differentiation of the fly on the same host species has not been documented. In contrast, de Jong *et al.* (2001) found no evidence for sympatric host formation using allozymes for a flea beetle with more than one sympatric host plant.

Until now only a single study (Michalakis *et al.* 1994) has investigated the genetic structure of both an insect and its host plant. We aim to address some of these gaps in the fields of comparative genetic structures, local adaptation and speciation by studying a species-specific, insect-plant mutualism from a population genetics and geographical perspective.

The host plant in this study is the carnivorous (Anderson and Midgley In press) plant family Roridulaceae with one genus and two species, *Roridula dentata* and *Roridula gorgonias*. Both species are found in allopatric, discrete, patchy populations (See Fig. 1). Distribution records of *R. dentata* suggest a disjunct distribution range, with populations more or less continuously distributed from the southern to the central part of the range. But a large gap in plant distribution is evident between the central and northern parts of *R. dentata*'s range. *Roridula* typically occurs in nutrient poor habitats (Obermeyer 1970) and plants augment the low soil nitrogen conditions by capturing insects using a sticky trap mechanism (Ellis and Midgley 1996). However the plants do not have digestive enzymes to digest prey and rely on carnivorous hemipterans to digest the prey for them (Ellis and Midgley 1996; Anderson and Midgley 2002). These insects then defecate on the plants' leaves and the plants absorb the digested nitrogen through their cuticles (Ellis and Midgley 1996). Each plant is associated with a species-specific hemipteran, *Pameridea marlothi* on *Roridula dentata* and *Pameridea roridulae* on *Roridula gorgonias* (Dolling and Palmer 1991). The hemipterans spend their entire life cycle on *Roridula* plants and cannot complete their life cycle without them (unpublished data). Plants are

pollinated primarily by juvenile *Pameridea* which are flightless and hence do not move much from plant to plant (Anderson *et al.* In press). As a result, gene flow between plant populations is predicted to be low.

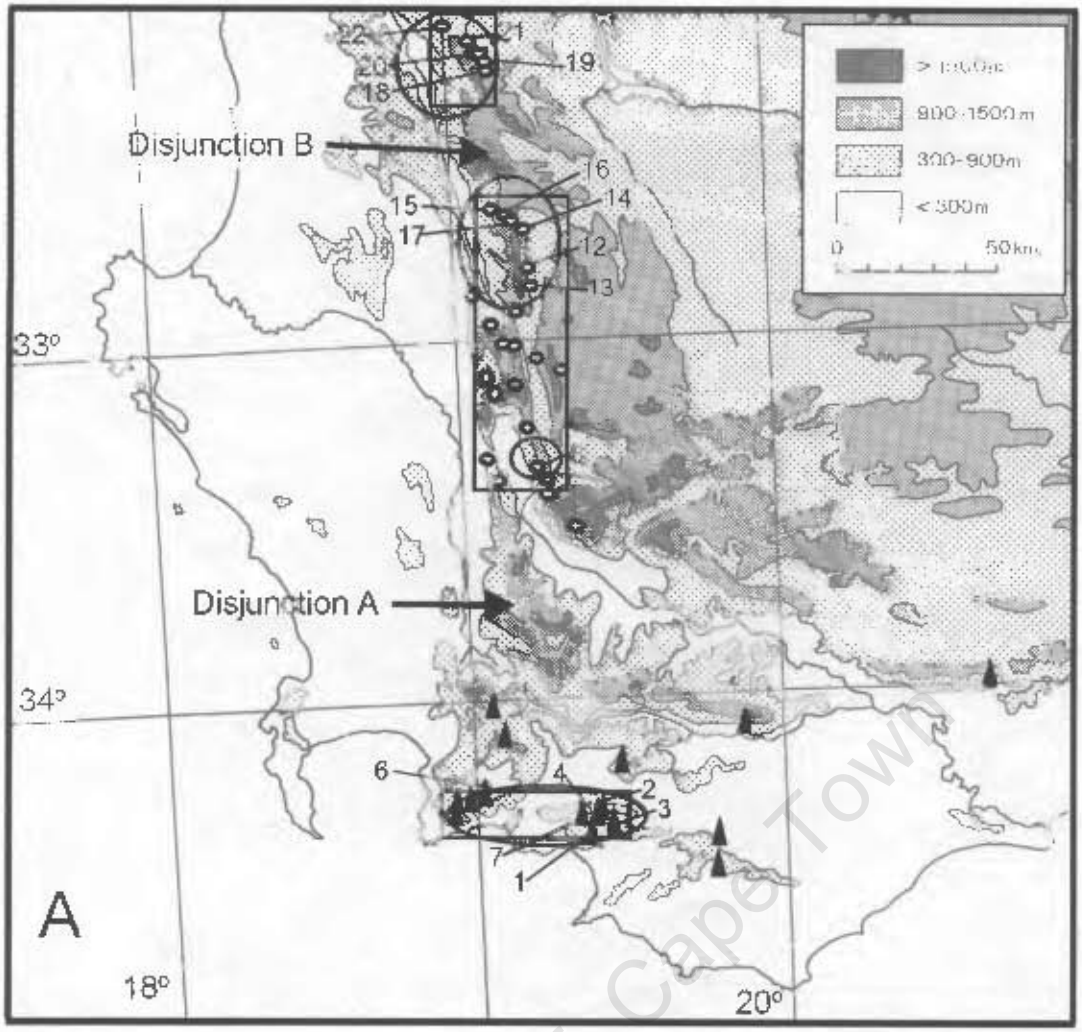
We examined the genetic structure of the two obligate mutualistic hemipterans and their host plants. As populations are discrete and isolated, it is expected that significant genetic subdivision at the population level may be observed for both hemipterans and plants. Because the gene flow in plants is predicted to be low, we hypothesise that plant populations are likely to be genetically and possibly phenotypically different, enabling adaptive deme formation to occur. Since there is a species-specific, obligate mutualistic relationship between plants and insects (Anderson and Midgley 2002, Anderson *et al.* In Press), we expect that the genetic patterns between plants and insects may be concordant. By relating gene flow and speciation patterns to the geographic structure of mutualists, we also examine the effects of host plant spatial distribution and hemipteran dispersal characteristics on speciation in these taxa.

METHODS

Collection and electrophoresis

In order to determine the distributions of *Roridula* species, collection data was recorded from the Bolus herbarium (University of Cape Town) and Compton herbarium (Kirstenbosch Botanical Gardens). In addition, new localities were sampled after conservation officials, farmers and biologists provided distribution records. Between 1999 and 2001, we sampled both *Pameridea* and *Roridula* from as many localities as we could find. Several localities remained unfound, either due to recent local extinctions or incomplete locality data. Seed material was collected from 11 *R. dentata* localities (Pop8, Pop15, Pop16, Pop17, Pop12, Pop14, Pop22, Pop18, Pop20, Pop21, See Fig 1.) and six *R. gorgonias* localities (Pop1, Pop7, Pop3, Pop2, Pop6, Pop4). *Pameridea* were collected from nine *R. dentata* localities (Pop8, Pop15, Pop16, Pop12, Pop14, Pop13, Pop22, Pop18, Pop20) and five *R. gorgonias* localities (Pop1, Pop7, Pop3, Pop2, Pop6). The discrepancy between the numbers of hemipteran and plant sites sampled is due to a paucity of hemipterans at some sites and refrigeration problems. Seeds were not collected from the site Pop13 because plants were too young to bear flowers. If plant populations were smaller than 20 individuals, then one capsule was collected from each plant in the population. In larger populations, single capsules from 20 to 24 plants were collected. In large populations, capsules were haphazardly picked along two perpendicular, bisecting transects from within half a meter of the transect line. The

Figure 1. Map of the South Western Cape (South Africa), showing known *Roridula* and *Pameridea* populations (they are exactly the same). Circles represent *R. dentata-P. marlothi* populations and triangles represent *R. gorgonias-P. roridulae* populations. Populations that are numbered were used in the genetic analysis. Two significant gaps in the distribution range of *Roridula* are labelled "disjunction A" and "disjunction B." Major UPGMA clades are mapped as circles (*Roridula*) and squares (*Pameridea*).



distance between samples depended on the population size. Adult hemipterans were collected in a similar fashion. However in small *Roridula* populations more than one hemipteran was collected per plant. Seeds were placed on ice and live hemipterans were taken back to the laboratory where they were both frozen at -80°C. Whole hemipterans were homogenised in 0.01 M Tris buffer (pH 8), using a glass rod attached to a variable-speed electric motor. Samples were used within four weeks of being collected and they were centrifuged for 5 min at 12 000 $R.min^{-1}$ prior to use. In contrast, the endosperm of the *Roridula* seeds was dissected out and only this material was homogenized using the vegetative extraction buffer I from Cheliak and Pitel (1984). Filter paper wicks (Whatmans # 3) were dipped into the supernant of centrifuged samples and these were inserted into 13 % horizontal starch gels. The sample tissue of the whole bugs and the plant endosperm was very small and a maximum of two gels were run per sample and we were unable to obtain a complete genotype of all loci for each individual. Complete genotypes for each individual are not necessary for most population genetic analyses. However assignment tests are analyses on the level of the individual and for these tests it is important to analyse several loci per individual. Most importantly, for the plants, care was taken not to use more than one seed per capsule for each locus investigated.

In *Roridula*, the enzymes MDH (E.C. 1.1.1.37), ADH (E.C. 1.1.1.1), GPI (E.C. 5.3.1.9) and DIA (1.6.-.-) were resolved on a continuous Histidine-citrate buffer system, pH 6.0 (Stuber *et al.* 1977). Pep LGG (E.C.3.4.-.-), IDH (E.C. 1.1.1.42), ME (E.C. 1.1.1.40) and PGM (E.C. 5.4.2.2) were resolved using a discontinuous Tris-citrate-borate-lithium hydroxide buffer: with a gel buffer pH 8.7 and an electrode buffer pH 8.0 (Ridgeway *et al.* 1970). G6 (1.1.1.49) and MPI (E.C. 5.3.1.8) were resolved using a continuous Tris-borate-EDTA buffer system , at pH 8.6 (Markert and Faulhaber 1965).

In *Pameridea* MPI, AK (E.C. 2.7.4.3), ME, G6 were also resolved on a continuous Tris-borate-EDTA buffer system at pH 8.6 (Markert and Faulharber 1965). The enzyme SOD (E.C. 1.15.1.1) was also resolved while staining for MPI on the above buffer system. The enzymes PGD (E.C. 1.1.1.44), MDH, ACN, DIA and IDH were all resolved using a continuous Tris-citrate buffer system (Whitt 1970). The discontinuous Tris-citrate-borate-lithium hydroxide buffer system (Ridgeway *et al.* 1970) was also used to resolve the enzymes PGM, EST (E.C. 3.1.1.1), GPI, HEX (E.C. 2.7.1.1) and ARK for *Pameridea*.

Statistical analysis

Allele and genotype frequencies were calculated using the Genepop program (Raymond and Rousset 1995). Genotype frequencies were tested for deviation *from* Hardy-Weinberg equilibrium using a χ^2-goodness- of-fit test.

The genetic structure among populations was tested by Fisher's exact test and the sequential Bonferroni-type correction (Rice 1989) was applied whenever necessary to correct for significance in these multiple tests. Wright's F-statistics F_{st} were estimated according to Weir and Cockeram (1984) using the computer program Genepop (Raymond and Rousset 1995). In addition, because *R. dentata* and *P. marlothi* have a wide distribution, gene flow in these species were also analysed at a regional level. Populations were broadly categorized as belonging to either one of three regions, namely north, central and south (See figs. 1 and 2). The average pairwise F_{st} was calculated for each region and also for population pairings between each region and species.

The mean unbiased (Nei 1978) genetic distance was calculated for each pairwise comparison of populations. Using the UPGMA clustering algorithm (Sneath and Sokal 1973) we mapped major population clusters in order to determine whether populations tend to cluster according to geographic proximity.

Assignment tests were performed to assess the population of origin in *Pameridea* individuals. These tests were performed according to the method of Rannala and Mountain (1997) using the Gene Class package (Cornuet *et al.* 1999). The test assumes Hardy-Weinberg equilibrium within each population and hence it was not performed on *Roridula* where no population was in Hardy Weinberg equilibrium. Unfortunately hemipterans are very small and the complete set of genotypic data was not available for each individual. Hence only individuals with data for seven polymorphic loci or more were used for this analysis. This left 167 individuals from nine populations for *P. marlothi* and 89 individuals from five populations for *P. roridulae*.

To relate the dispersal capabilities of *Roridula* and *Pameridea* to geographic distributions we plotted F_{st} between all pairwise combinations (see above) with the corresponding pairwise geographic distances. Correlations were made using the Mantel permutation procedure (Mantel 1967) associated with the Spearman rank correlation coefficient as test statistics using the program Genepop (Raymond and Rousset 1995). We also repeated the analysis after excluding the single geographically outlying population (pop8) to determine if relationships were due to this population alone. Although pairwise $F_{st}/(1-F_{st})$ is sometimes considered a more

appropriate measure for correlations with distance (Rousset 1997), we could not accurately apply this statistic as we had some F_{st} values of one, rendering the above calculation meaningless. To justify using F_{st}, we made the "corrected" correlations of $F_{st}/(1-F_{st})$ by substituting any value of $F_{st} = 1$ with the next highest F_{st} value in the analysis. Correlation coefficients and P values of genetic and geographic structures were tabulated for comparative purposes using F_{st}, "corrected $F_{st}/(1-F_{st})$" and also Nei's genetic distance.

RESULTS

Roridula

Locus Polymorphism

Eight of the nine loci examined in the six *R. gorgonias* populations were monomorphic (Table 1a). Only the locus MDH-1 was polymorphic in this species and two alleles were detected at this locus. The allele MDH-1^B was fixed in study sites Pop7, Pop6 and Pop1, occurred at low to moderate frequencies in Pop3 (0.063) and Pop2 (0.523), and was absent in Pop4 (Table 1a).

Six (G6-1, LGG-2, ME-1, PGM-1, IDH-2 and MDH-1) of the 12 loci examined in the 11 *R. dentata* populations were monomorphic (Table 1a). At most four alleles were detected per polymorphic locus. Most local populations were fixed. Out of 77 population/locus combinations, only eight were polymorphic, six of which corresponded to northern populations and two from the central region. Of the nine loci resolved for both *Roridula* species, two were monomorphic (LGG-2 and ME-1), with the same alleles being shared by both species. There were fixed differences between the species at all of the remaining seven loci (Table 1a).

Table 1a. *Roridula* allele frequencies

	R. gorgonias						*R. dentata*										
							South	Central					North				
Locus	Pop1	Pop7	Pop3	Pop2	Pop6	Pop4	Pop8	Pop15	Pop16	Pop17	Pop12	Pop14	Pop22	Pop19	Pop18	Pop20	Pop21
LGG-1																	
(N)	13	14	10	13	13	17	5	18	22	16	18	4	21	6	23	30	5
A	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000	1.000	1.000	1.000	1.000	0.000	0.000	0.000	0.000	0.000
B	1.000	1.000	1.000	1.000	1.000	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
C	0.000	0.000	0.000	0.000	0.000	0.000	1.000	0.000	0.000	0.000	0.000	0.000	1.000	1.000	1.000	1.000	1.000
GPI-1																	
(N)	13	15	10	13	12	16	5	18	21	17	18	4	20	6	23	31	5
A	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.870	0.000	0.000
B	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000	1.000	1.000	1.000	1.000	0.000	0.000	0.000	0.000	0.000
C	0.000	0.000	0.000	0.000	0.000	0.000	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.188	0.130	1.000	1.000
D	1.000	1.000	1.000	1.000	1.000	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
E	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000	0.813	0.000	0.000	0.000
ADH-1																	
(N)	13	15	10	13	13	16	5	18	22	17	18	4	20	6	20	35	5
A	0.000	0.000	0.000	0.000	0.000	0.000	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
B	1.000	1.000	1.000	1.000	1.000	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
C	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
DIA-1																	
(N)	13	15	10	13	13	16	5	18	22	16	18	4	20	6	21	30	5
A	0.000	0.000	0.000	0.000	0.000	0.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.875	1.000	0.933	1.000
B	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.125	0.000	0.067	0.000
C	1.000	1.000	1.000	1.000	1.000	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
PGM-2																	
(N)	17	16	16	16	14	16	5	21	19	19	20	6	16	6	19	24	5
A	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000
B	1.000	1.000	1.000	1.000	1.000	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
C	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000	1.000	1.000	1.000	1.000	0.938	0.950	1.000	1.000	0.000
D	0.000	0.000	0.000	0.000	0.000	0.000	1.000	0.000	0.000	0.000	0.000	0.000	0.063	0.050	0.000	0.000	0.000
G6--1																	
(N)	10	11	11	17	11	11	5	17	15	18	11	6	11	6	17	10	5
A	1.000	1.000	1.000	1.000	1.000	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

B	0.000	0.000	0.000	0.000	0.000	0.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
MDH-1																	
(N)	18	16	16	22	14	16	6	22	20	19	21	6	16	6	18	24	5
A	0.000	0.000	0.000	0.000	0.000	0.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
B	1.000	1.000	0.063	0.523	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
C	0.000	0.000	0.938	0.477	0.000	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
MPI-1																	
(N)	0	0	0	0	0	0	6	20	15	18	17	3	14	6	17	15	5
A	NA	NA	NA	NA	NA	NA	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
B	NA	NA	NA	NA	NA	NA	0.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
IDH-1																	
(N)	0	0	0	0	0	0	6	19	19	20	18	6	13	6	16	22	5
A	NA	NA	NA	NA	NA	NA	0.000	0.895	0.368	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
B	NA	NA	NA	NA	NA	NA	1.000	0.105	0.632	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
LGG-2																	
N	13	14	10	13	13	17	5	18	22	16	18	4	21	6	23	30	5
A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
ME-1																	
N	17	16	16	16	14	16	6	21	19	19	20	6	16	6	19	24	5
A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
IDH-2																	
N	0	0	0	0	0	0	6	19	19	20	18	6	13	6	16	22	5
A	NA	NA	NA	NA	NA	NA	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000

Table 1b. *Pameridea* allele frequencies

	P. roridulae					*P. marlothi*								
						South	Central					North		
LOCUS	Pop1	Pop7	Pop3	Pop2	Pop6	Pop8	Pop12	Pop13	Pop15	Pop16	Pop14	Pop22	Pop18	Pop20
MPI-1														
(N)	30	19	30	24	20	20	20	20	20	20	15	25	21	20
A	0.000	0.000	0.000	0.000	0.000	0.075	0.000	0.000	0.000	0.000	0.167	0.120	0.048	0.025
B	1.000	1.000	1.000	1.000	1.000	0.925	0.925	1.000	0.925	1.000	0.833	0.880	0.952	0.975
C	0.000	0.000	0.000	0.000	0.000	0.000	0.075	0.000	0.075	0.000	0.000	0.000	0.000	0.000
PGD-1														
(N)	20	14	13	20	20	20	15	9	20	15	16	8	15	22
A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.875	0.867	0.818
B	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.125	0.133	0.182
PGM-2														
(N)	24	20	32	20	24	20	19	20	20	30	15	32	21	20
A	0.000	0.000	0.000	0.025	0.042	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
B	0.000	0.000	0.000	0.000	0.000	0.050	0.026	0.025	0.000	0.000	0.000	0.000	0.000	0.000
C	0.000	0.000	0.000	0.000	0.000	0.950	0.974	0.975	1.000	1.000	1.000	0.000	0.024	0.000
D	0.125	0.025	0.000	0.050	0.125	0.000	0.000	0.000	0.000	0.000	0.000	1.000	0.976	1.000
E	0.875	0.950	1.000	0.925	0.833	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
F	0.000	0.025	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
ME–1														
(N)	15	19	10	35	20	40	25	20	20	40	15	23	25	30
A	1.000	1.000	1.000	1.000	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
B	0.000	0.000	0.000	0.000	0.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
MDH-1														
(N)	20	25	30	15	30	40	20	10	25	25	15	28	28	33
A	1.000	1.000	1.000	1.000	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
B	0.000	0.000	0.000	0.000	0.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
ACN-1														
(N)	26	37	15	31	25	20	19	19	33	20	15	23	15	23
A	0.000	0.000	0.000	0.000	0.000	0.225	0.053	0.000	0.045	0.000	0.000	0.000	0.000	0.065
B	0.962	1.000	1.000	1.000	1.000	0.775	0.711	0.842	0.758	0.950	1.000	1.000	1.000	0.935
C	0.038	0.000	0.000	0.000	0.000	0.000	0.237	0.158	0.197	0.050	0.000	0.000	0.000	0.000
AK–1														
(N)	15	15	20	30	15	20	19	10	15	20	17	30	44	35
A	1.000	1.000	1.000	1.000	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
B	0.000	0.000	0.000	0.000	0.000	0.975	0.842	0.950	0.633	0.925	0.706	0.000	0.159	0.000
C	0.000	0.000	0.000	0.000	0.000	0.025	0.158	0.050	0.367	0.075	0.294	1.000	0.841	1.000
DIA-1														
(N)	30	39	15	18	40	0	0	0	0	0	0	0	0	0
A	0.000	0.000	0.000	0.000	0.063	NA	NA	NA	NA	NA	NA	NA	NA	NA
B	0.950	0.872	1.000	0.833	0.900	NA	NA	NA	NA	NA	NA	NA	NA	NA
C	0.050	0.128	0.000	0.167	0.038	NA	NA	NA	NA	NA	NA	NA	NA	NA
EST-1														
(N)	36	15	40	34	23	20	15	15	19	15	14	20	21	20
A	0.000	0.000	0.000	0.000	0.000	0.000	0.067	0.000	0.079	0.000	0.000	0.000	0.000	0.000
B	0.000	0.000	0.000	0.000	0.000	0.050	0.067	0.033	0.079	0.067	0.214	0.175	0.048	0.050
C	0.042	0.000	0.000	0.088	0.022	0.950	0.800	0.700	0.816	0.800	0.786	0.525	0.667	0.750
D	0.903	0.900	1.000	0.868	0.957	0.000	0.000	0.167	0.026	0.000	0.000	0.300	0.286	0.175
E	0.056	0.100	0.000	0.044	0.022	0.000	0.067	0.100	0.000	0.133	0.000	0.000	0.000	0.025
G6–1														
(N)	37	13	35	24	20	15	19	16	19	20	27	25	21	22
A	0.054	0.000	0.000	0.021	0.050	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
B	0.000	0.000	0.000	0.000	0.000	0.133	0.053	0.000	0.026	0.100	0.019	0.000	0.048	0.068
C	0.000	0.000	0.000	0.000	0.000	0.867	0.947	1.000	0.974	0.900	0.981	1.000	0.952	0.932
D	0.946	1.000	1.000	0.979	0.950	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
GPI														
(N)	40	20	31	36	41	20	20	25	20	35	27	40	35	34
A	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.038	0.014	0.015
B	0.000	0.000	0.000	0.000	0.000	0.975	0.950	1.000	0.950	0.914	1.000	0.000	0.000	0.000
C	0.000	0.000	0.000	0.000	0.000	0.025	0.050	0.000	0.050	0.086	0.000	0.962	0.986	0.985
D	0.063	0.100	0.000	0.042	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

E	0.887	0.900	1.000	0.903	0.915	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
F	0.050	0.000	0.000	0.028	0.085	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
G	0.000	0.000	0.000	0.028	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
HEX														
(N)	23	20	35	20	20	20	20	20	26	35	17	15	15	20
A	0.000	0.000	0.000	0.000	0.000	0.000	0.025	0.000	0.038	0.000	0.000	0.000	0.000	0.000
B	0.000	0.000	0.000	0.000	0.000	1.000	0.975	1.000	0.962	1.000	1.000	1.000	1.000	1.000
C	1.000	1.000	1.000	1.000	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
IDH 1														
(N)	21	36	14	20	24	20	19	19	31	24	15	19	14	21
A	0.310	0.264	0.321	0.175	0.208	0.000	0.026	0.000	0.000	0.000	0.033	0.000	0.000	0.000
B	0.500	0.625	0.571	0.500	0.604	0.825	0.526	0.658	0.484	0.417	0.467	0.526	0.643	0.714
C	0.190	0.111	0.107	0.325	0.188	0.150	0.395	0.316	0.435	0.479	0.467	0.447	0.286	0.214
D	0.000	0.000	0.000	0.000	0.000	0.025	0.053	0.026	0.081	0.104	0.033	0.026	0.071	0.071
IDH 2														
(N)	21	36	14	20	24	20	19	19	32	24	15	22	15	22
A	0.000	0.000	0.000	0.000	0.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
B	1.000	1.000	1.000	1.000	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
SOD														
N	23	20	35	20	20	20	20	20	26	35	17	15	15	20
A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
ARK														
N	40	20	31	36	41	20	20	25	20	35	27	40	35	34
A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000

Hardy-Weinberg equilibrium

Out of the 10 cases of polymorphism (two in *R. gorgonias* and eight in *R. dentata*), a single locus (PGM-2 in study site Pop19) was found to be in Hardy-Weinberg equilibrium ($P > 0.05$), but in this population both sample size and polymorphism were low. The exact probability test for di-allelic loci with small sample size did not confirm Hardy-Weinberg expectations at the two other loci for which this particular population was polymorphic ($P < 0.05$). When Hardy-Weinberg equilibrium was tested in all polymorphic loci examined, every population with resolved polymorphic loci was found to have a significant excess of homozygotes, thus differing from the Hardy-Weinberg null hypothesis ($P < 0.05$). In *R. dentata*, the estimate of F_{is} was large ($F_{is} = 0.94$) and statistically greater than zero when tested over all populations and all loci ($\chi^2 = 26.2$, $P < 0.05$). Similarly F_{is} for *R. gorgonias* was large and significantly greater than zero ($F_{is} = 0.92$, $\chi^2 = 30.4$, $P < 0.0001$).

Population structure

F_{st}

Other than three F_{st} pairings of zero within the central region, all pairwise F_{st} values, were significantly positive (Table 2a) for *R. dentata* and a single pairwise F_{st} (8.3 % of all comparisons) was not significantly different from zero for *R. gorgonias* (Table 2a). The paired F_{st} values were generally very high for both *Roridula* species. Those paired F_{st} values, which were less than 0.2 for *R. dentata*, generally corresponded to geographically close populations. However, despite their geographical proximity close populations sometimes had high and significant F_{st} values. For example, Pop15 and Pop16 are only 0.5 km apart but have a paired F_{st} of 0.4286 ($P < 0.0001$, Table 2a). Populations that were separated by distances greater than 40 km had F_{st} values very close to one. Similarly, *R. gorgonias*, F_{st} was lower than 0.2 ($F_{st} = 0.0001$) for the pair of Pop3 and Pop4 which are 0.5 km apart. However, other nearby *R. gorgonias* populations also had high, significant F_{st} values.

Genetic differentiation was very high at all regional levels investigated (Fig. 2). The seven fixed allele differences (with no allele in common) suggest a complete lack of gene flow between species and in concordance the average F_{st} pairings (calculated by averaging paired F_{st} values, where population pairs were from different species) between these two plant species was very high ($F_{st} = 0.98$) and the range in F_{st} was very small (Fig. 1). All F_{st} population pairings between *Roridula* species were highly significant ($P < 0.0001$).

There were also fixed differences between regions of *R. dentata* and also within regions. As a result, the average paired F_{st} values between different regions was

high (088-0.95 depending on the region) and the range in F_{st} was small (Fig. 2). See Table 2a for significance levels of individual population pairings. Population pairings within regions had high average F_{st} values although the range of F_{st} values within each region was high (from 0.00-1.00). Similar to the F_{st} pairings within regions for *R. dentata*, *R. gorgonias* also had a high average pairwise F_{st} with a large range of values (Fig. 2).

UPGMA and Map

The UPGMA of genetic distances for the 17 *Roridula* populations reveal two distinct groups, not surprisingly corresponding to the two species (Fig. 3). These two species are separated at a D-value of 1.49. Within the cluster formed by *R. dentata* populations, three sub clusters are clearly discernable: the single southern population separated from the other *R. dentata* populations at a D-value of 0.412 and the northern populations separated from the central populations at a D-value of 0.202. In addition to the three *R. dentata* groupings being genetically distinct, these three groupings are also geographically distinct and form three geographic clusters (Fig. 2).

Pameridea

Locus polymorphism

Nine of the 16 loci resolved for *P. roridulae* were monomorphic. In all polymorphic loci examined, a single allele was found in high frequencies for all populations (Table 1b). Thus there were no strong frequency differences between populations.

Five of the 15 loci resolved for *P. marlothi* were monomorphic (ARK, ME-1, MDH-1, SOD and IDH-2). A fifth locus (HEX^B) was almost monomorphic as a single allele (HEX^B) occurred at very high frequencies (> 0.962) throughout all populations. Three of the polymorphic loci resolved were diagnostic for regions and distinguished individuals of the northern populations from the remaining populations. For example, fixed differences were observed for the locus PGM-2 and GPI, distinguishing the northern populations from the rest. Strong frequency differences were also found for the locus AK, which also corresponded to the northern populations.

Fixed differences between the two *Pameridea* species were observed in 8 loci (53.3%) (PGM-2, ME-1, MDH-1, AK-1, G6-1, GPI, HEX, and IDH-2). Of the remaining loci, frequency differences were evident for the loci ACN-1 and EST-1.

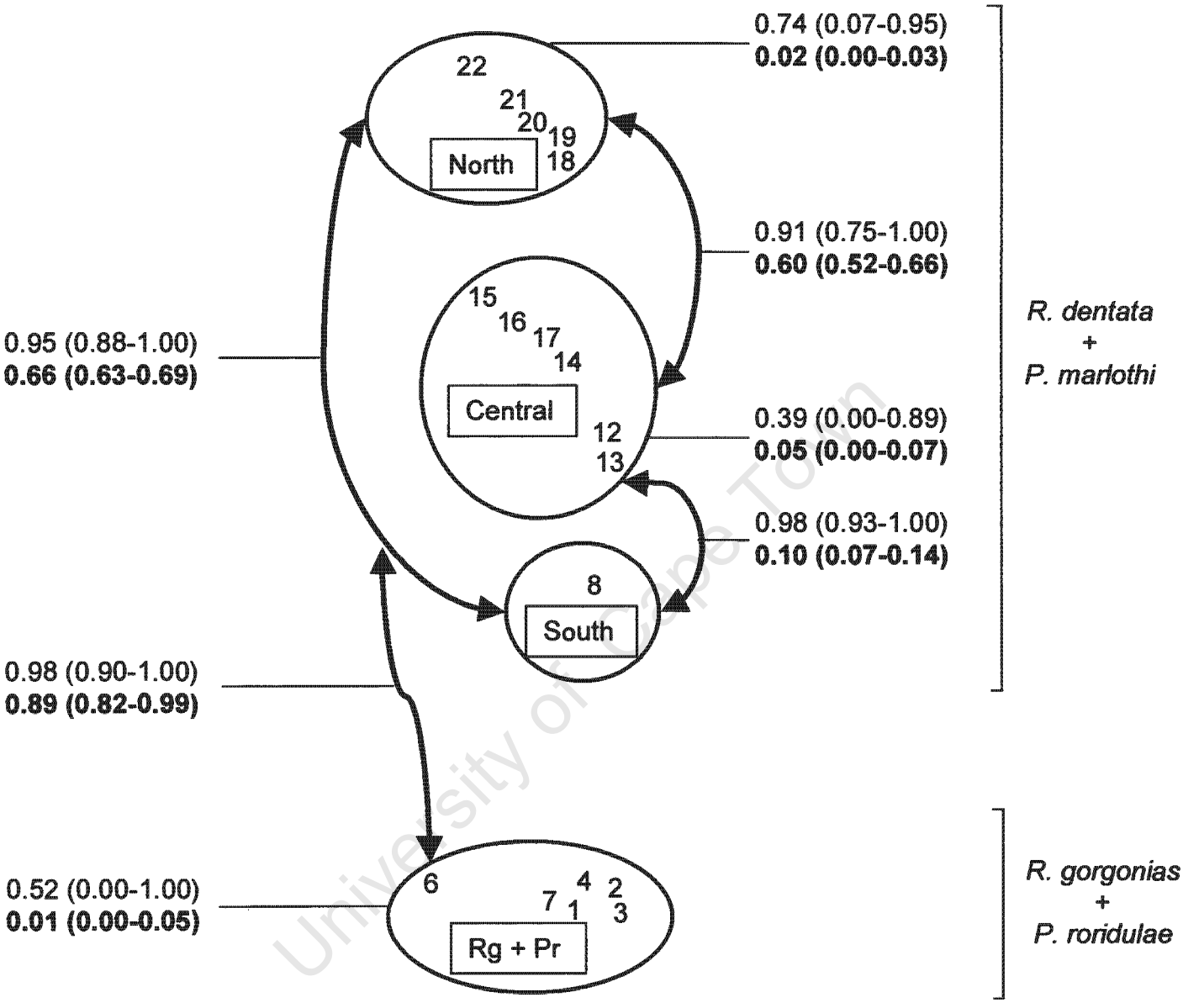


Figure 2. Average paired F_{st} values (range) for *Roridula* and *Pameridea*, where *Pameridea* values are in bold and *Roridula* values are in normal text. One species pair (*R. dentata* and *P. marlothi*) has been divided into three geographical regions (north, central and south). The other species pairing of *R. gorgonias and P. roridulae* (Rg +Pr) has a narrow distribution and hence is not divided into regions. We measure F_{st} pairings within regions, between regions and between species.

Table 2. Pairwise F_{st} (below diagonal), distance and significance above diagonal for *R. dentata*, separated into three regions (Table 2a), *R. gorgonias* (Table 2b), *P. marlothi* (2c) and *P. roridulae* (2d). P > 0.05 =ns, 0.01 < p < 0.05 = *, 0.001 < p < 0.01 = **, 0.0001 < p < 0.001 = ***, p < 0.0001 =****

2a

	South	Central					North			
	Pop8	Pop15	Pop16	Pop17	Pop12	Pop14	Pop22	Pop19	Pop18	Pop20	Pop21
Pop8	######	74****	74****	74****	59****	76****	138****	116****	116****	117****	134****
Pop15	0.9738	######	0.5****	0.9****	22****	4****	60****	52****	52****	52****	52****
Pop16	0.9298	0.4286	######	0.3****	23***	3*	60****	52****	52****	52****	52****
Pop17	1.0000	0.8916	0.3407	######	23 np	3 np	60****	52****	52****	52****	52****
Pop12	1.0000	0.8860	0.3258	0.0000	######	24 np	80****	63****	63****	63****	63****
Pop14	1.0000	0.8343	0.1958	0.0000	0.0000	######	58****	46****	46****	46****	46****
Pop22	0.9794	0.9417	0.8576	0.9724	0.9732	0.9551	######	13**	14****	11****	4****
Pop19	0.8770	0.8543	0.7481	0.8572	0.8626	0.7719	0.0746	######	2****	2****	7.5****
Pop18	0.9490	0.9169	0.8361	0.9319	0.9336	0.8989	0.8272	0.6347	######	2****	11****
Pop20	0.9636	0.9418	0.8758	0.9593	0.9610	0.9446	0.8875	0.6838	0.8102	######	7****
Pop21	1.0000	0.9621	0.8938	1.0000	1.0000	1.0000	0.9558	0.7673	0.8998	0.9005	######

2b

	Pop7	Pop6	Pop4	Pop3	Pop2	Pop1
Pop7	######	18 np	4.5****	5****	5****	2****
Pop6	0.0000	######	20.5****	21****	21****	19 np
Pop4	1.0000	1.0000	######	0.5 ns	0.8****	3.5****
Pop3	0.9333	0.9289	0.0000	######	0.3****	4****
Pop2	0.4108	0.3945	0.4585	0.3517	######	4****
Pop1	0.0000	0.0000	1.0000	0.9373	0.4260	#####

2c

	South	Central					North		
	Pop8	Pop12	Pop13	Pop15	Pop16	Pop14	Pop22	Pop18	Pop20
Pop8	#####	59**	54**	74***	74**	76***	135****	121****	122****
Pop12	0.0669	#####	4 ns	22 ns	23 ns	24	80****	63****	63****
Pop13	0.0747	0.0096	#####	24 ns	25 ns	26*	82****	67****	67****
Pop15	0.1227	-0.0016	0.0569	#####	0.5 ns	4	60****	52****	52****
Pop16	0.1132	0.0179	0.0354	0.0512	#####	3**	60****	52****	52****
Pop14	0.1456	0.0376	0.0739	0.0173	0.0426	#####	58****	46****	46****
Pop22	0.6888	0.5983	0.6580	0.5662	0.6379	0.6109	#####	14 ns	11 ns
Pop18	0.6273	0.5470	0.6013	0.5214	0.5899	0.5777	0.0113	#####	2 ns
Pop20	0.6752	0.6012	0.6602	0.5741	0.6439	0.6285	0.0363	-0.0003	#####

2d

	Pop1	Pop7	Pop3	Pop2	Pop6
Pop1	#####	2 ns	4**	4 ns	19 ns
Pop7	0.0019	#####	5**	5 ns	18**
Pop3	0.0143	0.0174	#####	0.3****	21***
Pop2	0.0043	0.0091	0.0517	#####	21 ns
Pop6	-0.0060	0.0082	0.0248	0.0084	#####

Hardy-Weinberg equilibrium

Out of the 86 cases of polymorphism, only four (4.65 %) were found to be out of Hardy-Weinberg (HW) equilibrium, corresponding to the error wise rate. After a Bonferroni correction for multiple comparisons, no departure from panmixia was observed. Pooling of multi-allelic genotype frequencies for small sizes confirmed HW for individual locus/population combinations. In *P. marlothi*, F_{is} was not statistically different from zero (F_{is} = 0.036) when tested over all populations and all loci (χ^2 = 38.9, P > 0.05). Similarly F_{is} for *P. roridulae* was not significantly different from zero (F_{is} = 0.108, χ^2 = 31.5, P > 0.05).

Population structure

F_{st}

In *P. marlothi*, pairwise F_{st} values were low within regions (Tables 2c and 2d, F_{st} = 0 to 0.07) and many population pairs within regions were not significantly different. In contrast, all pairings between regions were significantly different. For *P. roridulae*, pairwise F_{st} values were very low within the distribution range sampled (F_{st}= 0 to 0.05).

Several fixed allele differences between the two *Pameridea* species suggest a complete lack of gene flow and this is also reflected by the high average paired F_{st} (F_{st} = 0.98) between the two species (Fig. 2). At the intraspecific level, the average pairwise F_{st} between the southern and central *R. dentata* populations is comparatively low (F_{st} = 0.10) with relatively little variation, suggesting consistently good gene flow between these regions (Fig. 2). In contrast, high average F_{st} values were observed between northern and southern populations and also between northern and central populations (F_{st} = 0.66 and 0.60 respectively). Within *R. dentata* regions, the average F_{st} values were also low with low variation, suggesting consistently good gene flow between populations (Fig. 2).

The UPGMA (Fig. 3) of genetic distances for the 14 *Pameridea* populations also reveal two main groups, corresponding to the two species. These two clusters separated at a D-value of 0.943. The cluster corresponding to *Pameridea marlothi* can further be separated to form two distinct clusters, one grouping the northern populations, the other the central populations as well as the unique southern one (see Figs. 1 and 3). The northern populations diverged from the other *P. marlothi* populations at a D-value of 0.202. The southern population is separated from the central populations at a D-value of 0.012 (an order of magnitude lower than the next highest grouping) for this reason we have grouped the central and southern populations in figure 1.

Assignment tests indicated that of the 167 *P. marlothi* individuals (Fig. 4a), only 58.1% (97 individuals) were correctly assigned to their population of origin. The only population to have 100% of individuals correctly assigned was Pop8. This was the only population sampled from the southern region. Most individuals that were not assigned to their population of origin were either assigned to the nearest population (26 individuals or 15.6 %) or to another population within that region (39 individuals or 23.4 %). Only three individuals (1.8 %) were assigned to another region. These individuals were from study site Pop16 in the central range and were assigned to the study site Pop8 in the south. A similar scenario was evident for *P. roridulae* (Fig. 4b) where 52.8% (47/89 individuals) were correctly assigned to their population of origin. Most other individuals were assigned to another population in the same region (31 individuals or 34.83%) and the remainder were assigned to the nearest population (20 individuals or 22.47 %). For *P. roridulae*, no individuals were assigned to other regions, as the species does not commonly occur over a large enough area to warrant splitting it into regions.

Correlating genetic structures of Roridula *and* Pameridea

In *R. gorgonias*, there was no significant correlation between geographic distance and pairwise genetic distance (Table 3) and the Mantel statistic was insignificant for all measures of genetic distance (e.g. r = 0.03, P > 0.2 for F_{st}). There were no significant correlations either between *Pameridea roridulae* and geographic distance (Table 3). Finally, no significant correlation was found between the pairwise genetic distances of *P. roridulae* and the corresponding genetic distances of *R. gorgonias* (e.g. r = 0.22, P > 0.2 for F_{st}, Table 3).

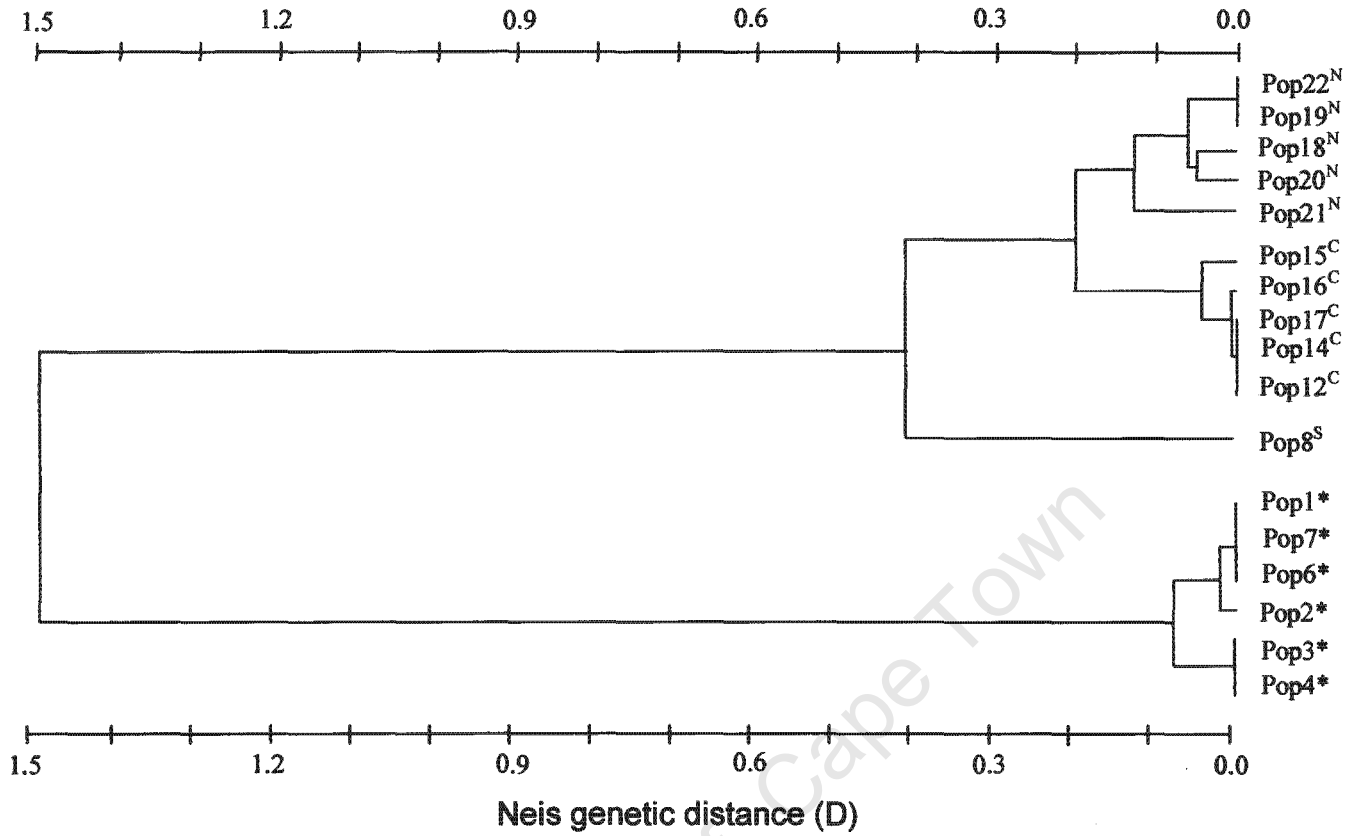
In contrast, *R. dentata* populations had positive and significant correlations with geographic distance for all measures of genetic distance (e.g. r = 0.509, P < 0.0005, for F_{st}, Table 3, Fig. 5a). Positive and significant correlations of all measures of genetic distance versus geographic distance were also found for the hemipterans *P. marlothi* that live on *R. dentata* (e.g. r = 0.7095, P < 0.0005, Table 3, Fig. 5b). However, a point of note is that there is a large "step" in the genetic pattern in this species due to higher than expected F_{st} values for population pairings involving the northern populations. The correlations of *R. dentata* and *P. marlothi* genetic distances were also significant for all measures of genetic distance however both the Mantel P values and correlation coefficients were lower than for correlations between geographic and genetic distance (Table 3, Fig 5c).

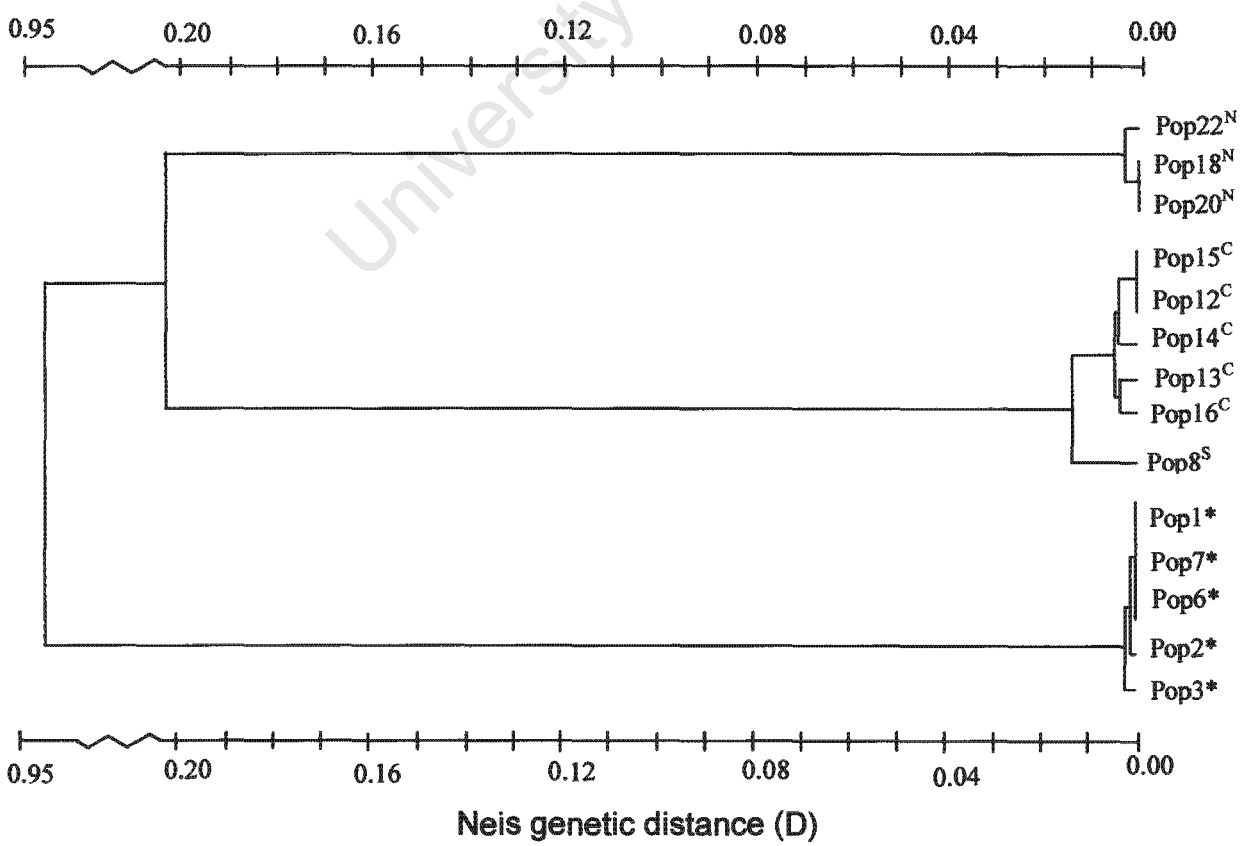
Dropping the outlying population (Pop8) from the analyses did not affect the broad outcome as all correlations remained significant and positive (Table 3). However,

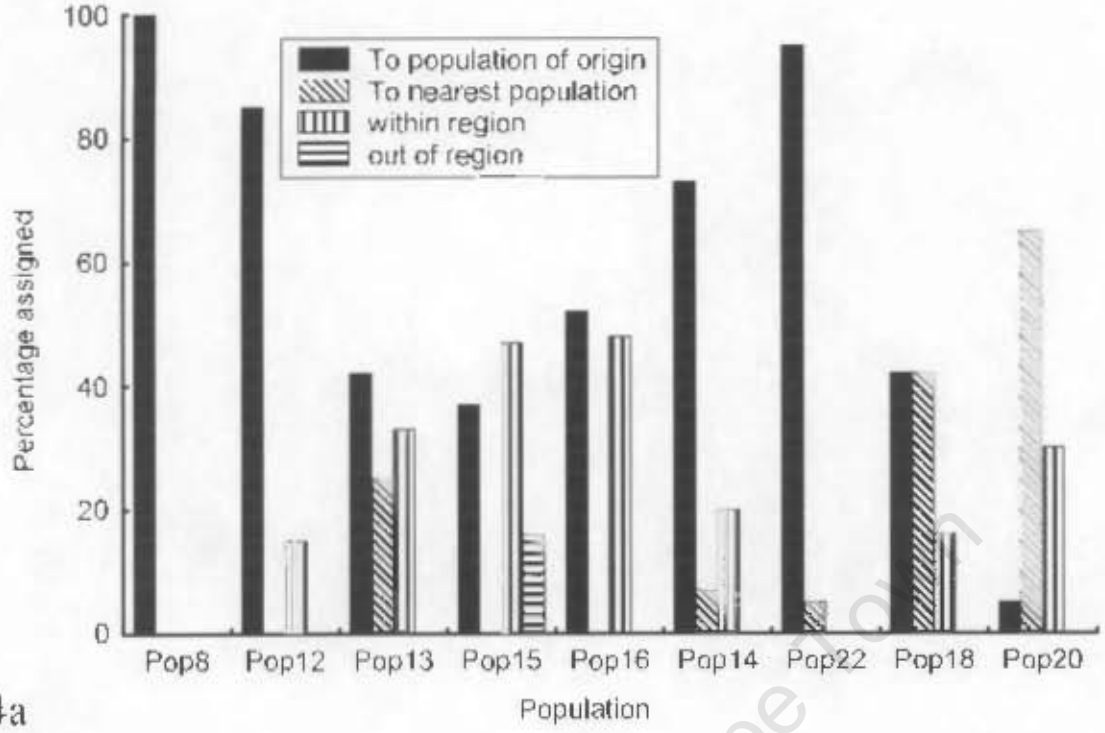
dropping population 8 from the analyses effectively increased correlation coefficients and decreased significance in all accounts.

Table 3. Correlation coefficients and P values using the Mantel test. Correlations were made between paired genetic distance and geographic distance for both *Roridula* and *Pameridea*. Correlations were also made for paired genetic distances between *Roridula* and *Pameridea*. We show correlations using three measures for genetic distance (Nei, Fst and either Fst/(1-Fst) or "corrected" Fst/(1-Fst). The letter "A" represents correlations involving either one of the species pair *R. gorgonias or P. roridulae*. "B" and "C" represent correlations involving the other species pair (*R. dentata* or *P. marlothi*. "B" is inclusive of all populations whereas "C" represents analyses where population 8 has been excluded.

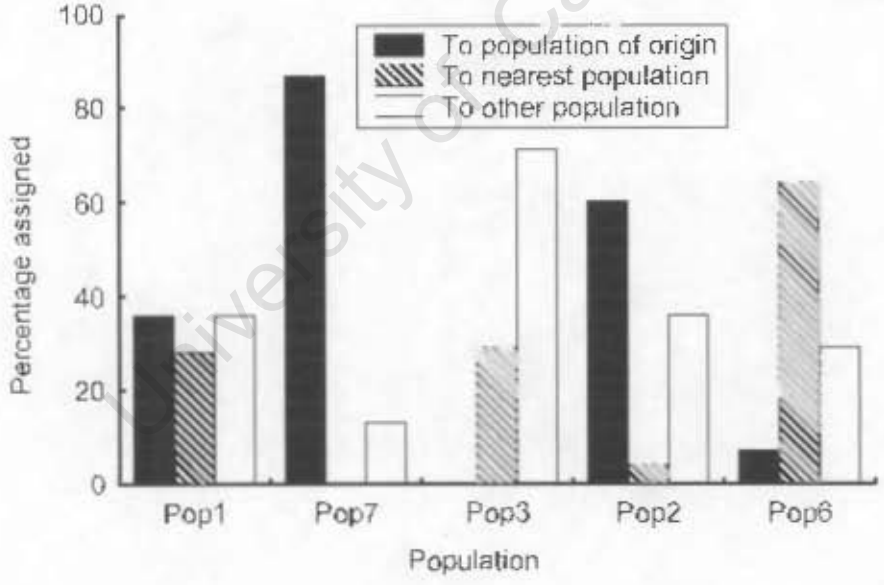
		Roridula vs distance		*Pameridea* vs distance		*Pameridea* vs Roridula	
		Mantel-P	r	Mantel-P	r	Mantel-P	r
A	Nei	0.084	0.002	0.7286	-0.1159	0.2244	0.2171
A	Fst	0.295	0,03	0.9194	-0.5184	0.096	0.3825
A	Fst/(1-Fst)			0.9144	-0.5149	0.0989	0.2257
A	corrected Fst/(1-Fst)	0.28	0.0871				
B	Nei	0.003	0.698	0.001	0.6914	0.00262	0.1036
B	Fst	0.00043	0,509	0.00041	0.7095	0.0035	0.4828
B	Fst/(1-Fst)			0.0004	0.7241		
B	corrected Fst/(1-Fst)	0.0002	0.6069			0.0022	0.2665
C	Nei	0.002	0.7494	0.0012	0.9315	0.0011	0.8965
C	Fst	0.001	0.518	0.0036	0.921	0.0024	0.5958
C	Fst/1-Fst			0.0043	0.9136	0.0016	0.6277
C	corrected Fst/1-Fst	0.005	0.5472				







4a

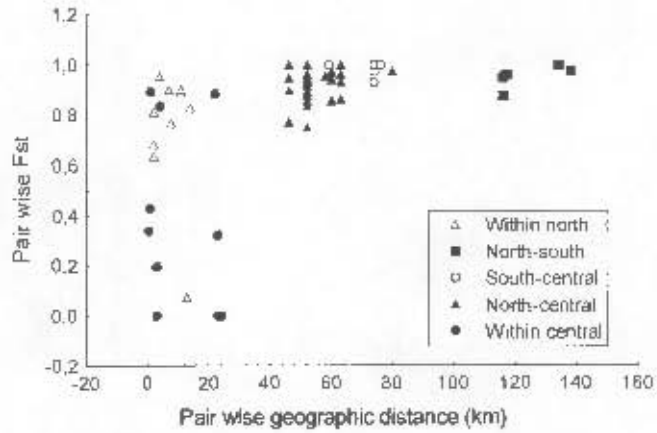


4b

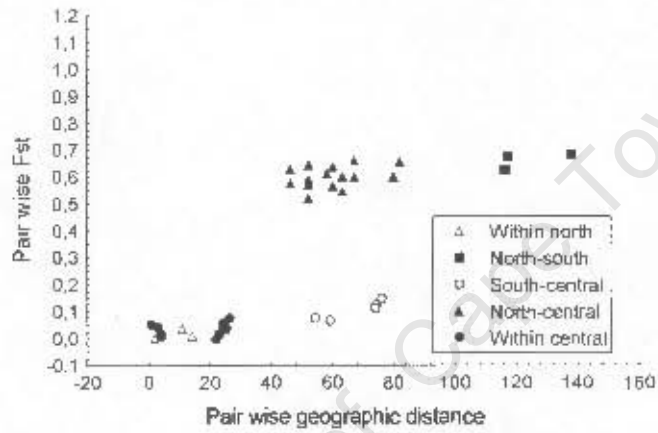
Figure 4a. The percentage of *Pamerídea marlothi* analysed from different populations, and assigned correctly to their population of origin or incorrectly to other *Pamerídea marlothi* populations.

Figure 4b. The percentage of *Pamerídea roridulae* analysed from different populations, and assigned correctly to their population of origin or incorrectly to other *Pamerídea roridulae* populations.

5a



5b



5c

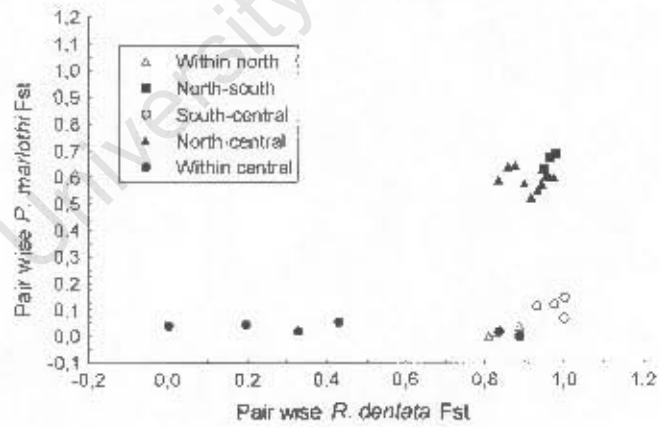


Figure 5a. Isolation by distance patterns for the host plant *R. dentata* using Fst as a measure of genetic distance.

Figure 5b. Isolation by distance patterns for the hemipteran *P. marlothi* using Fst as a measure of genetic distance.

Figure 5c. Correlation of plant (*R. dentata*) and hemipteran (*P. marlothi*) genetic structures using F_{st} as a measure of genetic distance.

DISCUSSION

Roridula

Population structure

Roridula populations are strongly subdivided at all levels. Subdivisions based on pairwise F_{st} values not only show strong divisions between species but also between close intraspecific populations confined to small regions. Thus it is likely that such populations may also exhibit phenetic differences to which *Pameridea* can adapt at local or regional scales. Both species of *Roridula* are characterized by strong deficits of heterozygotes in all populations with polymorphic loci. This is most likely caused by inbreeding as a result of high levels of self-pollination or local substructure due to low levels of dispersal (Wahlund effect). We view selfing due to lack of pollen dispersal as the most likely explanation for the departure from panmixia, because the genus is self-compatible and is pollinated by flightless juvenile *Pameridea* (Anderson *et al.* In press). Inbreeding is likely to promote the genetic and phenotypic divergence of *Roridula* populations.

The consistently high F_{st} values for *Roridula*, including some very close by populations, suggest that even limited gene flow between most populations is rare or non existent. As a result, the genetic patterns in this genus are probably an artefact of ancient or historical events. In support of this, De Meester *et al.* (2002) hypothesize that high genetic differences between nearby populations often reflects founder effects (i.e. historical colonization of new habitats rather than contemporary gene flow). It is likely that historical fragmentation events would cause similar patterns. Contemporary distribution patterns and gene flow are unlikely to have had a great impact on the genetic structure of *Roridula*, other than the fact that there may be some similarities between past and present distribution patterns. There are however a few examples of very low F_{st} values between some close *Roridula* populations and the most logical explanation for this is relatively recent founder effects from a common pool or recent vicariance events, although the existence of gene flow cannot be ruled out. Although only a single polymorphic locus was resolved for *R. gorgonias*, the species nevertheless also appeared very subdivided. It is likely that better resolution and similarly strong subdivision would have been found if more polymorphic loci were resolved and a larger area was sampled.

Pameridea

Population structure

As with *Roridula* plants, the division of *Pameridea* into two separated, distinct species based on morphological characters (Dolling and Palmer 1991) was confirmed by allozymes. Fixed allele differences at several loci clearly indicate no

gene flow and thus reproductive isolation. Note however that, as the two plant (and insect) species are allopatric, it is unknown whether such reproductive isolation is due to intrinsic barriers or to geographic barriers. Within *P. marlothi*, hemipteran populations are also subdivided into two regions and a small number of geographically close populations are significantly differentiated. However, the hemipteran populations are not as strongly structured as plant populations and most geographically close populations are undifferentiated. This is most likely because of higher gene flow in the hemipterans due to their better dispersal capabilities. The genetic structuring of hemipterans is an important indicator that local adaptation is possible, although more likely at a regional level than a local population level. A very strong gene flow barrier exists that separates the northern hemipteran populations from all others. The distribution of allelic variation among the loci examined is consistent with other incipient insect species (Thorpe 1983, 1994). Thus, based on allelic variation, it is likely that northern and central/southern bugs are incipient species. It is most likely that this gene flow barrier is the result of the large disjunction found in the *R. dentata* distribution pattern. Since *Pameridea* is species-specific, distant *Roridula* populations need to be linked by "stepping stone" populations to facilitate hemipteran movement and gene flow. But northern populations, are highly isolated (Fig. 1) with no stepping stone populations between them and populations from other regions. This represents a gene flow barrier to *P. marlothi* and is probably responsible for the incipient speciation observed using allozymes. In contrast, despite the southern region being separated from the central region by large geographical distances, hemipteran gene flow is relatively high. The most plausible explanation for this is that several populations between these regions (Fig. 1) act as "stepping stones" for gene flow. As a result hemipterans from the central and southern regions form a single metapopulation. This is corroborated by the relatively low F_{st} values (a mean of 0.10 and a range of 0.07-0.14) between the southern and central populations, which are consistent with normal observations where the range in F_{st} usually varies from 0.01 to 0.20 (McCauley and Eanes 1987, Rodderick, 1996, Peterson and Denno 1998).

Assignment tests also suggest that good gene flow exists between populations within a region but that gene flow between regions is low. The only inter-regional gene flow suggested by this analysis was also between southern and central populations, which we believe is the result of "stepping stone" populations linking the two regions. Since strong gene flow seems to exist between populations within a region but not between isolated regions; it may be expected that adaptive deme formation would most likely occur at a regional level, especially if regions are isolated (Law and Koptour 1986). However, since individual plant populations within regions are genetically distinct, there may be no common genotype (and possibly phenotype) within regions that hemipterans are able to adapt to. Instead, the

genetic structure of *Pameridea* and *Roridula* seem to be most strongly affected by geographic structure and continuity of host plant distribution.

Correlating genetic structures of Roridula *and* Pameridea

There are no patterns of isolation by distance in *R. gorgonias.* However, this is to be expected, as a single locus was polymorphic, whereas several polymorphic loci were found in *R. dentata*, which has a much larger sampled range size. In addition, only six *R. gorgonias* populations were analysed. Although several polymorphic loci were resolved for *P. roridula*e, only five populations were analysed. In this species, no significant differentiation was found between populations, which were all less than 30 km apart. Hence, no isolation by distance was found in either *P. marlothi* or *R. gorgonias*. There was also no correspondence between the genetic distances of these two species, which may also be related to the paucity of polymorphic loci for *R. gorgonias*.

In contrast, isolation by distance trends in both *R. dentata* and *P. marlothi* suggest that mutualist populations are not panmictic and that genetic structure is strongly related to geographic structure. As a result of the very low gene flow in *R. dentata*, its present disjunct distribution pattern is unlikely to influence the genetic pattern in this species. In contrast, the genetic structure of *P. marlothi* populations is strongly affected by contemporary distribution patterns and this is because gene flow in this species is high provided there are no obstacles. The genetic structure of this species is strongly influenced by the highly isolated northern populations, which create a large "step" (population pairings with northern populations are higher than expected) in the correlation between genetic structure and geographic distribution. Gene flow between the southern and central region is comparatively high due to the presence of "stepping stone" populations.

Hence there is a discrepancy between the genetic structure of *P. marlothi* and *R. dentata* (Fig 5c) that results in comparatively poor correlations (albeit significant) of their genetic distances. The discrepancy between the genetic structures at this level also suggests that adaptive deme formation plays a secondary role to dispersal in determining genetic structure. Nevertheless, the fact that there is correlation between genetic structures of these two mutualists may encourage local adaptation to take place.

This study suggests that cospeciation has taken place in the *Pameridea-Roridula* complex and that cospeciation may be in progress in the northern *R. dentata* populations. Furthermore, allozyme results suggest that local adaptation at some level can potentially play a role in structuring mutualist populations, since the effects of "swamping" local adaptation by gene flow are likely to be minimized by the patchy host distribution and poor hemipteran dispersal across large discontinuities. Despite this, distribution patterns and different dispersal attributes most strongly influence the genetic structures of *R. dentata* and *P. marlothi*. In contrast to this study, other genetic studies (Mulvey *et al.* 1991, Michalakis *et al.* 1994, Martinez et al. 1999, Mutikainen and Koskela 2002) found no common structure between host and parasite genotypes. The lack of any genetic correlation between hosts and parasites in these studies may have been due to small distribution range (e.g. Mulvey *et al.* 1991), very good dispersal capabilities of parasites (e.g. Michalakis *et al.* 1994) or weak selective pressures.

In our study, plants and hemipterans have clearly different scales of gene flow throughout their range; where plant hosts are very strongly subdivided at the population level and hemipterans are weakly subdivided or not at all. The difference in genetic structuring between these two organisms is most probably due to differences in the breeding systems and dispersal capabilities of these two species. Delmotte *et al.* (1999) showed the opposite to our study in that the fungal pathogen (*Microbotryum*) is less genetically structured than its plant host (*Silene*). However, similar to us, they attribute this difference to the fact that the pathogen self-fertilizes and has low gene flow whereas the host-plant is an outcrosser with good gene flow. Other studies also indicate that no rule dictates that either hosts or parasites should be more genetically structured. For example Mutikainen and Koskela (2002) found that hosts were more genetically structured than their parasites whereas Martinez *et al.* (1999), and Jerome and Ford (2002a, 2002b) found the opposite. Differences in the genetic structures of hosts and parasites are most likely to be a result of gene flow patterns and breeding systems. When gene flow is much higher among parasites than their hosts, low rates of speciation are expected (Johnson *et al.* 2003). If the same rules apply to mutualistic systems, then this may explain the low species diversity in the *Roridula-Pameridea* complex.

Because *Pameridea* and *Roridula* have such different scales of gene flow, *Pameridea* are unlikely to adapt to plant phenotype at the population level. Only in isolated populations (as in the extreme north) are interregional gene flow patterns between plants and hemipterans more congruent. Such groups of isolated populations may represent evolutionary hotspots as hemipterans may be able to

adapt to plants at the regional level. These findings are in concordance with the geographic mosaic theory of Thompson (1994) who predicts that the geographic structure of closely associated organisms has a major effect on the coevolutionary process. Our study is similar to that of Althoff and Thompson (1999) who found that the parasitoid *Agathis* and its moth host (*Greya*) have incongruent patterns of genetic structure as assessed by genetic markers. Incongruent genetic structures may also be caused by the fact that the geographical scale at which the interaction evolves may be different for each species involved in the interaction (Althoff and Thompson 1999). However, this is likely to be strongly affected by the relative gene flow capabilities of associated organisms. Because of the incongruent genetic structures of *Agathis* and *Greya*, Althoff and Thompson (1999) predict that coevolutionary processes in this system are unlikely at the level of local populations.

By correlating the genetic structures of several species of parasitic chewing lice and their hosts (pocket gophers), Demasters and Hafner (1993) demonstrated that cospeciation is likely to have taken place between hosts and parasites. However, few studies have found correlated genetic structures at the sub-specific level. For example Mulvey *et al.* (1991) examined the genetic structure of deer and parasitic helminths (Mulvey *et al.* 1991). In this study, there was no correlation between isolation and distance in either the parasite or host populations, nor was there any relationship between the genetic structures of helminths and deer. Michalakis *et al.* (1994) compared the population genetic structure of a phytophagous insect (weevil) and its host plant (thistle). They too found no isolation by distance for the weevils (possibly because weevils are strong fliers), nor did they find correlated genetic structures between host and parasite. Martinez *et al.* (1999) examined comparative population structures of the parasitic cuckoo and its primary magpie host. They found that genotypes were correlated with geographic structure in both species. They did not however find correlations between the genotypes of the hosts and parasites. Dybdahl and Lively (1996) compared the genetic structures of a snail and its trematode parasite and found that "stepping stone" geographical distance was correlated with both host and parasite genetic distances. In addition they found that genetic distances of snails and helminths were also correlated. They suggested that this correlation might be due to similar patterns of dispersal or due to responses of parasites to their hosts. Perhaps the most convincing case of local adaptation having a profound effect in correlating the genetic structures of hosts and parasites may be found in the related studies of Jerome and Ford (2002a) and Jerome and Ford (2002b). Here they found no correlation between geographic distances in hosts or parasites. However, they did find correlated genetic structures between the host and parasite genotypes. They also found that parasite genotypes were much more structured than the genotypes of their hosts, suggesting that the parasitic genotype is affected by many more factors than just the host genotype.

The difficulty in determining what causes correlated genetic structures is a general problem with these kinds of studies, and the present paper is no exception: correlated genetic structures for supposedly neutral markers say something about dispersal and drift patterns, but nothing about the joint evolution of two partners. However, such correlated structures might be necessary for local co-adaptation or coevolution to occur.

This study of *Roridula-Pameridea* differs from previous analyses of host parasite genetic structure in that those dealt with antagonistic relationships while this study examines a mutualism. Our study adds to the small body of literature on host parasite genetic structures by showing that similar principles apply in the coevolution and local adaptation of mutualisms. We show that genetic patterns of mutualists are strongly influenced by their distribution patterns and those of their partners, adding to the rapidly growing literature on the geographical mosaic theory (Thompson 1994). The different scales of gene flow in these two mutualist species also suggest that local adaptation is less likely on a local scale but may be promoted at a regional scale.

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9 Is genetic sub-structuring in *Pameridea* due to fine scale host tracking?

ABSTRACT

Correlated genetic structures have been found for a mutualist and its host suggesting that local adaptation may be promoted in this system. I determine whether adaptation to host plants is evident in this system and at what scale it has taken place. In choice experiments, the hemipteran (*Pameridea*) has a strong preference for its carnivorous host plant (*Roridula*) above unrelated host plants. Although *Pameridea* can live on other hosts, they seldom reach adulthood, their life expectancy is diminished and they fail to reproduce successfully. *Pameridea* prefers its host species to its closely related sister species (choice experiments). However, *Pameridea* does not exhibit intraspecific preferences towards plants from their natal populations above plants from non-natal populations. Nor are there any differences in *Pameridea* fitness on natal versus non-natal host plants. Although I do not find evidence for local adaptation, adaptation at the specific level is likely to accelerate cospeciation in this mutualism. At this level of divergence, *Pameridea* genotypes may be able to track those of its host plant.

INTRODUCTION

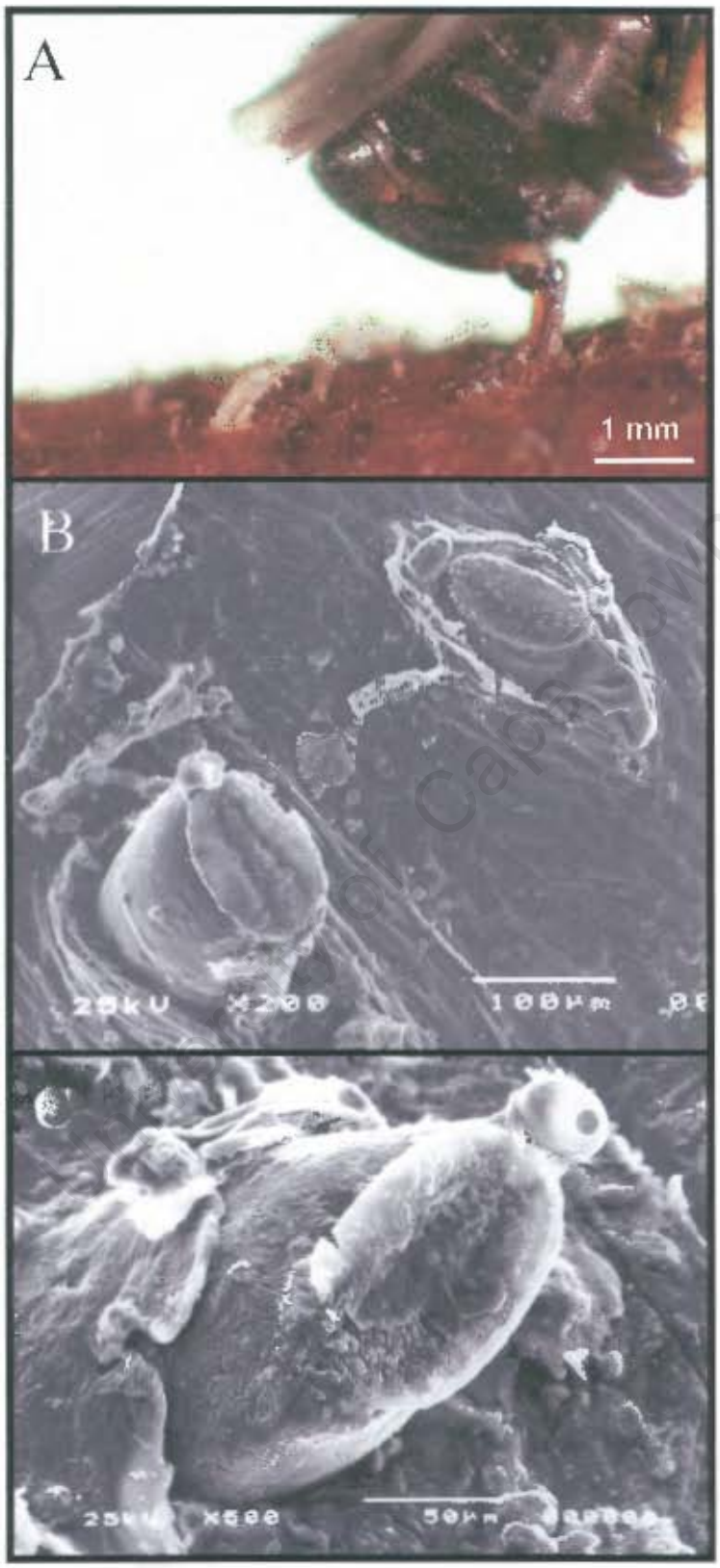
Phytophagous insect populations may become locally adapted to their host species (Scriber 1983) and in extreme examples, insects may become adapted to individual host plants (Karban 1989). The ability to adapt at such fine scales may be related to parasites having very short life spans compared to their hosts (Mopper 1996), and host-parasite relationships being frequently very specialized and intimate (Thompson 1994). Furthermore, host plants are often very heterogeneous in their chemical composition (Zangerl and Berenbaum 1993, Berenbaum and Zangerl 1998) and by specializing at the fine scale, insects may be able exploit their environment more efficiently than generalist insects. Adaptations to host plants may manifest themselves as preference hierarchies of parasites for different hosts (i.e. parasites may prefer their natal host populations above non-natal host populations e.g. Singer *et al.* 1988, Thompson 1988). Genetic sub structuring should result if phytophagous insects adapt to, or track the intrinsic host traits (i.e. traits of an individual, which are under strong genotypic control e.g. Feder *et al.* 1990), this may accelerate coevolution and the processes leading to speciation. Alternatively, genetic sub- structuring may result if parasites adapt to the extrinsic features of their

hosts' phenotype (these are features that are controlled by environmental conditions - Alstad 1998). However it is also possible that the genetic subdivision of parasite populations is non-adaptive and that low parasite gene flow may allow variations to arise through genetic drift and founder effects (Alstad 1998). Genetic drift may strongly affect the genetic structure of parasites whose host populations are geographically isolated. Chapter 8 shows correlations between the genetic structures of mutualist species and also genetic substructuring in both species. However, it is difficult to ascertain whether correlations and substructuring are due to local adaptation to host phenotype, extrinsic factors or genetic drift.

Although several studies (e.g. McCauley and Eanes 1987, McPheron *et al.* 1988, Alstad and Corbin 1990) show local genetic differentiation in phytophagous insects, few show that variation is due to the tracking of host genotypes. However, at least one study suggests that leaf miners are adapted to the defensive chemistry of individual plants (Mopper *et al.* 1995). Other studies suggest that genetic structuring is a function of non adaptive genetic drift or extrinsic factors that are unrelated to host genotypes (Alstad 1998, Ayres *et al.* 1987). Irrespective of the causes, the formation of discrete parasite races may promote speciation of parasites and may be one of the mechanisms that account for the enormous diversity of phytophagous insects (e.g. McPheron *et al.* 1988, Feder *et al.* 1988, Thompson 1988). However it is important to determine whether genetic subdivision is governed largely by selective pressures of the hosts' genotype, environmental heterogeneity (unrelated to host genotype) or genetic drift.

We investigate specialization of a hemipteran (*Pameridea*), which has a very close, mutualistic association with a carnivorous plant called *Roridula* (Dolling and Palmer 1991, Ellis and Midgley 1996). *Roridula* captures prey using sticky leaves although it has no digestive enzymes to digest prey (Marloth 1925, Ellis and Midgley 1996). *Pameridea* consumes prey and defecates on *Roridula*'s leaves and in this way *Roridula* absorbs up to 70% of its nitrogen (Ellis and Midgley 1996, Anderson and Midgley 2002). *Pameridea* are also important pollinators of *Roridula* (Anderson *et al.* In press). Although this relationship is generally mutualistic, it is nevertheless intimate enough to expect adaptive host race formation. *Pameridea* completes its entire life cycle on *Roridula* and is not recorded on any other plant species (pers. obs., Dolling and Palmer 1991, Schuh 1995). Parasitic tendencies include *Pameridea* laying its eggs in the woody tissue of *Roridula* (plate 7) and sucking sap from *Roridula* (unpublished data). As a result of this parasitic element of their association, *Pameridea* probably possess adaptations to *Roridulas*' chemical defences. Two allopatric species of *Roridula* occur and each hosts a different *Pameridea* species (only two *Pameridea* species are known, Dolling and Palmer

Plate 7. A. *Pamerídea marlothi* lays her eggs by ovipositing in the woody tissue of *Roridula*. Note the hatched egg in the fore ground. **B, C**. SEM micrographs of *P. marlothi* eggs embedded in the woody tissue of *Roridula dentata*.


A
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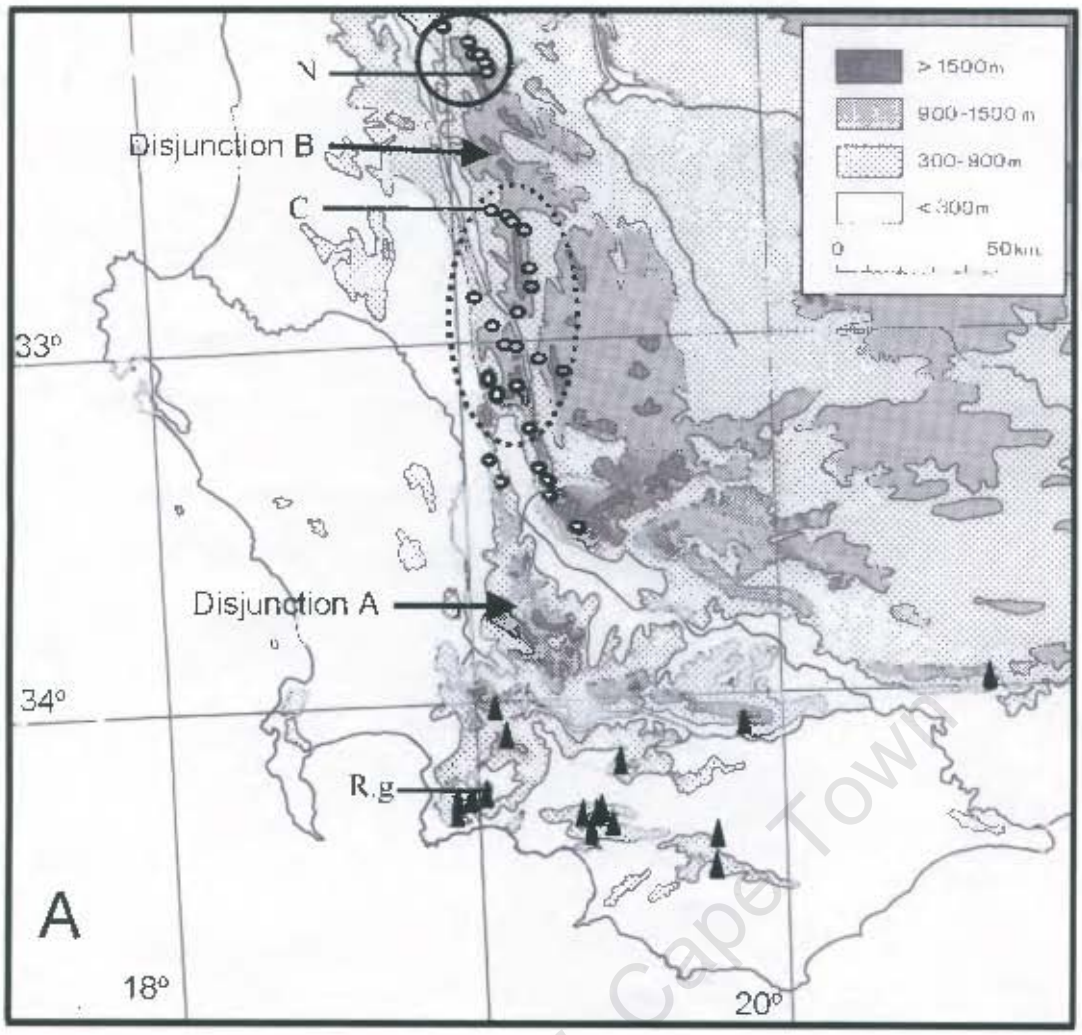
1991), suggesting that cospeciation has occurred. In addition, allozyme studies have revealed that individual *Roridula* populations are strongly differentiated due to very low geneflow and high rates of selfing (Anderson *et al.* In press). Genetic variation within *Roridula* populations is generally also low, suggesting that plants within populations are very similar (Anderson *et al* In press). If genetic variation relates to strong phenotypic variation, then it is possible that host tracking could occur in this system at the population level. In support of this, it was found that *Pameridea* is strongly sub-structured with some significant genetic differences between geographically close populations (chapter 8). However, this study also suggests that regional genetic differences between hemipteran populations are particularly strong and that adaptive host race formation is most likely to occur in the northern populations. These populations are geographically isolated (Fig. 1) and genetically differentiated enough to qualify hemipterans from this region as an incipient species based on allozyme data (chapter 8). Chapter 8 suggests that hemipterans are not able to traverse large distances between widely separated populations (with no stepping stone populations inbetween) because hemipterans are species specific and only moderately good fliers. As a result adaptation at the regional scale may be likely, especially for geographically isolated regions. Gene flow is also much greater for *Pamerideo* than for *Roridula* (chapter 8), which would also promote adaptation at the regional scale rather than the population scale.

If insects are locally adapted to particular host types, then I expect them to prefer and perform better on their own host populations/species in comparison to other host populations (Singer *et al.* 1988). Hence I investigate the degree and scale at which specialization has taken place in the *Roridula-Pameridea* mutualism by examining insect fitness on different hosts and host choice. This represents one of the only studies of local adaptation in a mutualism. I investigate whether *Pameridea* are able to survive and reproduce on commonly occurring non-carnivorous plant species. This has important implications on the ability of *Pameridea* to move between *Roridula* populations. I look at host choice by *Pameridea* to ascertain whether they have preferences for their natal plant species or population. Finally I compare fitness of *Pameridea* on natal and non-natal populations of *Roridula dentata*. Since *Pameridea* only occur on *Roridula* plants, it is expected that they may exhibit adaptations that allow them to recognise their host species over co-occurring, non-carnivorous species. If local adaptations to the hosts play a significant role in genetic substructuring of *Pameridea*, then I expect that fitness or preference hierarchies may be evident at the population or regional level.

Figure 1. Map of all known *Roridula* populations, showing the three populations used in the host choice study. *Roridula dentata* populations are represented by a northern (N) and a central (C) example whereas *R. gorgonias* populations are represented by the population marked "R.g."



METHODS

Interspecific adaptations

Roridula dentata cuttings, *Leucadendron salignum* (a common, non-carnivorous plant in the Proteaceae family) cuttings and plastic plants were all placed in a 1/4 strength Hoagland's solution (Hewitt 1966) and their aerial parts were covered with a fine mesh which effectively stopped the movement of insects to and from individual plants. Juvenile hemipterans were removed from the *Roridula* plants over the course of a four-week period. Once all juvenile hemipterans were removed, all plant cuttings were reseeded with ten first instar *P. marlothi* juveniles each. Five treatments (two cuttings per treatment) consisted of *Roridula* cuttings fed with one fly per week, *Roridula* cuttings without flies, *Leucadendron* cuttings with one dead fly placed on the leaves each week, *Leucadendron* cuttings with no flies and a plastic plant with no flies. Every three days, the total number of surviving hemipterans on each cutting were counted and the number of adult hemipterans were noted. Plants were kept for three months to ascertain whether oviposition and reproduction had been successful.

Host choice

Parasite preference for different host plants has been shown to be a genetically inherited and correlated with parasite performance (Singer *et al.* 1988). Therefore parasites that are physiologically adapted to a particular host plant, may preferentially choose that host plant above others. I examined preference in *Pameridea* to determine whether it preferred its natal host species or host population above others. Plants from three study sites were used including one *R. gorgonias* site, inhabited by the hemipteran *P. roridulae* and two geographically separated *R. dentata* populations inhabited by *P. marlothi*. *Roridula gorgonias* cuttings, *Leucadendron* (a common, non-carnivorous Proteaceae) cuttings and *P. Roridula* were also collected from the same site (Pop6, Fig. 1). This study site is approximately 100 km from the nearest *R. dentata* population and a disjunction exists in *Roridulas* range ensuring that *R. gorgonias* and *R. dentata* are geographically separated by about 70 km (Fig. 1). *Roridula dentata*, *Leucadendron* and *P. roridulae* were collected from one central population (Pop15, fig. 1) and one northern population (Pop18, Fig. 1). The northern population was separated from the central population by approximately 50 km with no known *Roridula* populations in-between (Fig. 1). Hemipterans from the northern and central *R. dentata* populations represent genetically distinct, incipient species and *Roridula* from these two regions are also genetically distinct.

Equal numbers of male and female *P. roridulae* were made to choose between a *R. gorgonias* cutting from their natal population and from a *R. dentata* cutting of similar proportions. Choices were also made between *R. gorgonias* cuttings (natal population), *Leucadendron* cuttings and a plastic plant. Prior to choice experiments, plant cuttings were kept for three weeks in a 1/4 strength Hoagland's (Hewitt 1966) nutrient solution in the absence of any hemipterans. All prey items on *Roridula* plants were removed. Choices were made by "blacking out" the outside of an aquarium and placing two cuttings (host plant and non-host) inside the aquarium, 15 cm apart. A single *Pameridea* was placed in a small container exactly between the two plants and the hemipteran was allowed to crawl onto the lip of the container and choose either plant to move onto. The aquarium was placed in a dark room (27°C) with a single light source shining directly above the hemipteran container (between the choice plants). A fine mesh net was placed over the top of the aquarium. Every half-hour, the aquarium was checked to ascertain whether the hemipteran had made a choice. If no choice was made after 1.5 hours, the hemipteran was removed and replaced. After a choice had been made, the aquarium was wiped down with alcohol and the positions of the two host plants were swapped to eliminate the possibility of hemipterans following each other's tracks. Plant specimens were replaced after five choices. Forty *P. marlothi* and 40 *P. roridulae* were tested in this way for each possible pairing (except for *P. marlothi* choices between *R. dentata* and *R. gorgonias* where 80 choices were made). Similarly, 40 *P. marlothi* were made to choose between *R. dentata* from their natal population, *Leucadendron* and plastic plants. In addition, *P. marlothi* from the northern population were made to choose between *R. dentata* from the natal population and *R. dentata* from the central population. *Pameridea marlothi* from the central population (n=40) were also made to choose between *R. dentata* from their natal population and *R. dentata* from the northern population.

Intraspecific adaptations

A more finely tuned experiment, testing whether *Pameridea* is locally adapted at the regional scale examined hemipteran fitness on *Roridula dentata* plants from natal and non-natal populations. Methods used were similar to those in the broad scale fitness study (see above). However, in this study, there were four treatments: *R. dentata* cuttings from the northern population (n = eight) with hemipterans from the northern population (three males, four females), *R. dentata* cuttings from the northern population (n = eight) with hemipterans from the central population (three males, four females), *R. dentata* cuttings from the central population (n = eight) with hemipterans from the central population (three males, four females), *R. dentata* cuttings from the central population (n = eight) with hemipterans from the northern

population (three males, four females). Hemipterans from all four treatments were fed with one fly per week.

RESULTS

Interspecific adaptations

The average life expectancy of *Pameridea* differed significantly between treatments ($F = 34.39$, $P < 0.000001$, ANOVA). The highest average life expectancy (SE) was found on *Roridula* plants where hemipterans were fed with flies (74.75 ± 7.28 days, Fig. 2). Hemipterans that were not fed on flies were still able to survive for approximately one month on either *Roridula* or *Leucadendron* plants, presumably feeding on plant sap alone (Fig. 2). Hemipterans on *Leucadendron* had longer lifespans when fed with flies than if they were raised on either *Leucadendron* or *Roridula* without flies ($P < 0.001$, Tukey HSD). *Pameridea* on plastic plants had the shortest life expectancies (2.15 ± 0.21 days). *Pameridea* only reached adulthood in two different treatments. Seventy percent reached adulthood on *Roridula* plants that were fed with flies. Although *Pameridea* reached adulthood on *Leucadendron* fed with flies, only 25 % managed to do so. Finally, a second generation of juvenile *Pameridea* only emerged on *Roridula* that were fed with flies. Over 95 juveniles were counted from the second generation. No second-generation juveniles were found on any of the other four treatments.

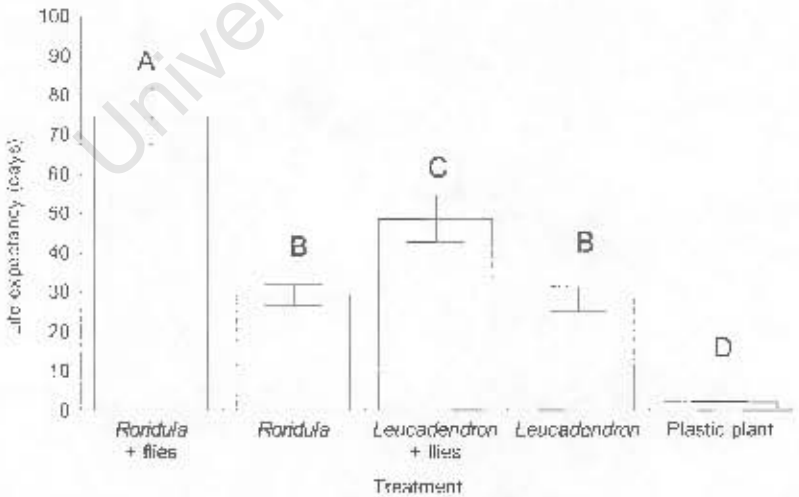


Figure 2. Mean life expectancy (SE) of first instar *P. roridulae* larvae after being placed on different plants. The hemipterans were given extra food (flies) on some hosts but not others. Different letters signify significant differences between treatments (Tukey HSD test, $P < 0.05$).

Pameridea roridulae chose their host plant, *R. gorgonias* above *R. dentata*, the plastic plant and the *Leucadendron* control (Fig. 3). Similarly *P. marlothi* also displayed preferences towards their species *R. dentata* above all other choices (Fig. 4). However, when faced with the choice of *Roridula dentata* from their natal population versus non-natal *R. dentata*, neither *P. marlothi* from northern nor from the central populations chose their natal plants preferentially (Fig. 5).

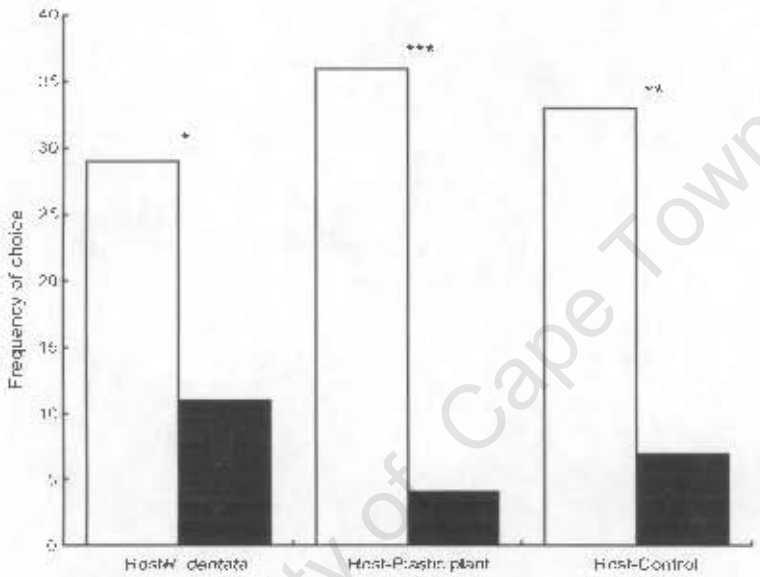


Figure 3 The frequency of choices made by *P. roridulae* for either the host plant (*R. gorgonias*) or non-host species. Choices for the host plant are clear bars and choices for the non-host plant are dark bars. Statistical significance (Fisher's exact test) is indicated by the symbols: * = 0.01 < P < 0.05, ** = 0.001 < P < 0.01, *** = 0.0001 < P < 0.001, **** = P < 0.0001.

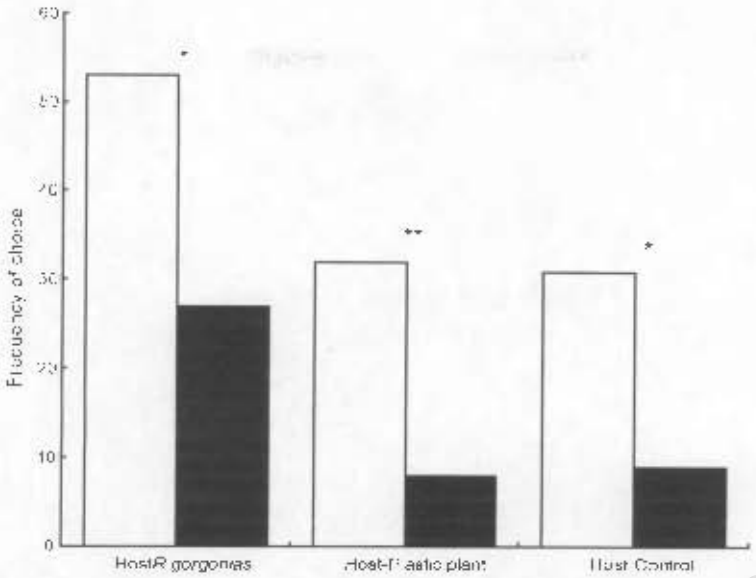


Figure 4. The frequency of choices made by *P. marlothi* for either the host plant (*R. dentata*) or non-host species. Choices for the host plant are clear bars and choices for the non-host plant are dark bars. See 2a for significance details.

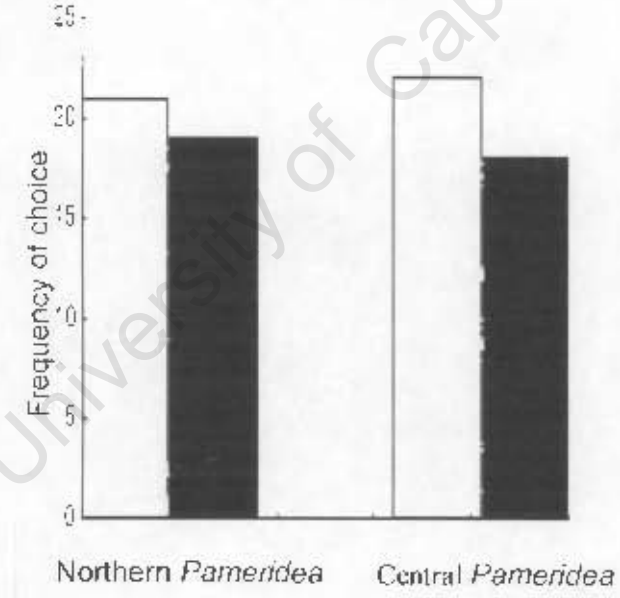


Figure 5. Intraspecific host choices made by *P. marlothi* from two different populations (north and central). Clear bars represent choices for the natal *R. dentata* population and dark bars represent choices for the non-natal *R. dentata* population. Choice comparisons were all non-significant using the Fisher's exact test ($P > 0.05$).

Natal or non-natal host plants made no difference to the number of juveniles recruited into the next generation (χ^2 = 0.507, P = 0.917, Kruskal-Wallis). Hemipterans from the northern population were equally fit on plants from the north (natal population) or central region (Fig 6). Similarly, hemipterans from the central population were equally fit on natal and non-natal plants (Fig 6).

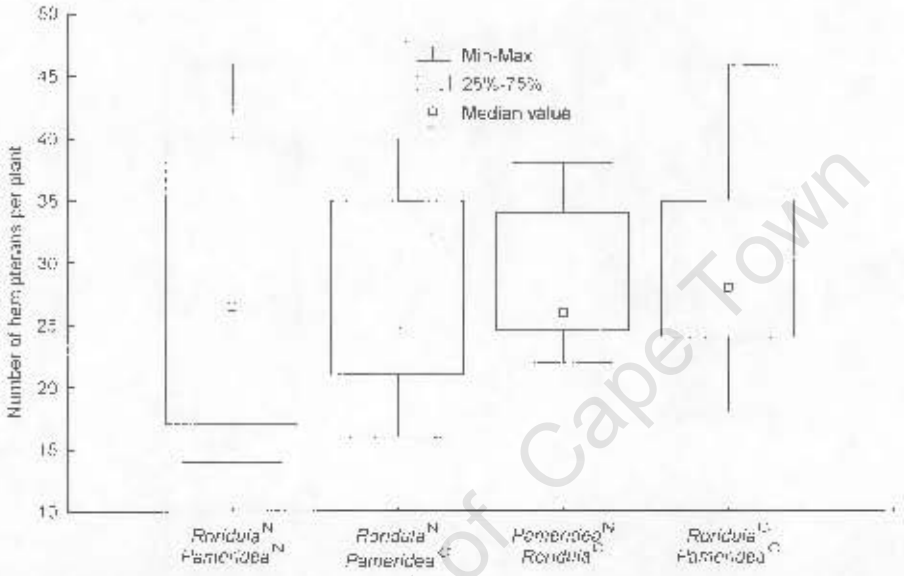


Figure 6. The number of hemipterans on natal and non-natal *R. dentata* plants after four months. Plants and hemipterans came from two populations (north and central), denoted by ^{N} and ^{C} respectively.

DISCUSSION

This study suggests that despite fine scale genetic structure of hosts and parasites (chapter 8), there is no evidence for local specialization, even at the regional scale. However I did find interspecific specialization suggesting that host tracking may occur once hosts have significantly diverged. Life expectancy data suggests that *Pameridea* can survive on co-occurring, non-carnivorous plants for long periods. Life expectancy on plastic plants is comparatively low, suggesting that hemipterans are able to gain nutrition from the plant sap of non-carnivorous plants. Life expectancy and chances of reaching adulthood are increased if *Pameridea* has access to insect food. The ability to survive for long periods on non-host plants may be essential for the recolonization of *Ronidula* populations, which are extremely patchy and sometimes far apart. By surviving on the plant sap of non-carnivorous plants, *Pameridea* is allowed sufficient of time to move between and colonize

Roridula populations, which would be unlikely if their lifespan (in the absence of *Roridula*) was only a few days (like that on plastic plants). Although *Pameridea* are able to survive on *Leucadendron* for long periods, very few reached adulthood and reproduction did not occur. Even though *Pameridea* can survive on non-carnivorous plants, it must still be regarded as a obligate and species-specific as it is unlikely to reproduce on non-carnivorous plants. Although sample size of non-carnivorous plants is very small, there are no genera, which are closely related to *Roridula* and I suspect that if *Pameridea* could reproduce on other species then it would have been recorded on other plants as well. Due to the fact that there are no close relatives to *Roridula* in the fynbos, there were no strong candidates for control plants. *Leucadendron* was chosen in this case due to the fact that it is numerically abundant and because it belongs to one of the dominant plant families in the area (Proteaceae).

Adaptations to a specialized life on *Roridula* plants include specialized host recognition systems of *Pameridea*. *Pameridea* is able to detect, and preferentially choose *Roridula* plants over co-occurring, non-carnivorous species. This may help in host recognition during recolonization events. Both species of *Pameridea* are also able to distinguish between the two *Roridula* species, even though they never occur in sympatry. I attribute this to strong differences in plant phenology although this study does not distinguish between visual or olfactory differences. Differences in plant phenology (at the interspecific level) may have developed during the speciation process as a result of genetic drift or natural selection and host tracking is likely to have resulted in the ability of *P. marlothi* to distinguish between the two species. Although *Pameridea* never needs to distinguish between different *Roridula* species in the wild, this ability may have developed as a side effect of evolving efficient host recognition systems. Incipient *Pameridea* species from the north and central regions did not preferentially choose plants from their population of origin. This may be because the experimental procedure is not fine-tuned enough to pick up such subtle differences. Alternatively differences in plant phenology are not large enough at this taxonomic level to elicit adaptive responses in *Pameridea*.

To test which of these is responsible for the lack of intraspecific choice; I looked at fitness on and off natal populations. These data suggest that fitness is the same on natal and non-natal *R. dentata* populations, and that *Pameridea* is not adapted to phenotypic differences in *Roridula* at the intraspecific level. I suspect that phenotypic differences at the intraspecific level are not great enough to induce strong selective pressures on *Pameridea*. Lack of adaptation may be due to lower antagonism amongst mutualists. The clonal nature of *Roridula* may also reduce antagonism between these two species. The lack of local adaptation is unlikely to be due to north-central "swamping" of adaptations because chapter 8 suggests that

there is no geneflow between the northern and central populations. However strong hemipteran geneflow from other close but phenotypically different *Roridula* populations may induce a swamping effect. The genetic differences between the northern and central *Pameridea* populations are probably the result of either genetic drift or other selective factors such as community structure or climate. Selective pressures induced by the host plants are only likely to cause divergences in *Pameridea* once plants are extremely different (i.e. different species). My results are very similar to the results of Reed and Hafner (1997) who found no local adaptation at sub-specific levels of chewing lice to their pocket gopher hosts. However, they did find that lice were adapted at the specific and generic level. Their study suggests that differences in gopher phenology are only great enough to cause divergence in lice once gopher populations have already diverged significantly (i.e. speciated). I suspect that both chewing lice and *Pameridea* may diverge significantly due to genetic drift or extrinsic factors before host tracking has any effect on parasite genotype. However host tracking is likely to reinforce species boundaries by causing further divergence after speciation has already taken place.

Similar to the studies of Alstad 1998 and Ayers *et al.* 1987, I suggest that in this system, fine and regional scale gene genetic structure is not due primarily to adaptations to host phenotype. Genetic sub structuring may have resulted from extrinsic environmental factors (e.g. climate and community structure) in this system. For example, Mopper *et al.* (1995) found that apparency to predators differed on natal and non-natal plants and was an important factor governing parasite fitness. The two *R. dentata* populations in this study have very different species-specific predator communities and this may contribute to extrinsic variation in this system (pers. obs.). This study only examined the effects of host plant genotype on *Pameridea* fitness and therefore, there may be scope to look at extrinsic factors in future. Alternatively, genetic drift can play an important role in fine and medium scale genetic structure of this system. Before completely ruling out local or regional adaptation in this system, it is necessary to perform reciprocal translocation experiments on a larger number of different *Roridula* populations. It may also help to examine a greater number of fitness traits in *Pameridea* (e.g. mass, age to maturity).

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SUMMARY

The aims of this study were to: (1) establish whether the *Roridula-Pameridea* mutualism is an obligate, one-on-one mutualism. Furthermore I examined some of the factors that are perceived to constrain the evolution and accelerate the collapse of obligate, one-on-one mutualisms. These included determining whether (2) the exploitative nature of mutualism causes conflict in this system, (3) the addition of an extra trophic level creates instability in the reward quality of the system. (4) Whether habitat fragmentation results in reciprocal genetic erosion. Lastly (5), I intended to establish which processes were predominantly responsible for the speciation process in this system and determine at what spatial scales they are acting.

1. *Pameridea* are important in maintaining the high natural seed set observed in *Roridula*. In addition, they facilitate the uptake of the majority of plant nitrogen. Without *Pameridea*, the characteristically small *Roridula* populations would most likely collapse as a consequence of reduced growth rates and fecundity. I suggest that *Roridula* is obligately dependent on *Pameridea*. Obligacy is also assumed for *Pameridea* as they are only found in association with *Roridula* in the field and fail to reproduce on other plants in the greenhouse. The relationship is specialized to the extreme (single mutualist partners) for both partners and hence this mutualism can be classified as an obligate, one-on-one mutualism.

2. Similar to other mutualisms, there is potential for conflict between *Pameridea* and *Roridula* based on differing plant performance under changing hemipteran densities. The negative plant performances under high hemipteran densities suggest that sap sucking by *Pameridea* is detrimental to *Roridula* and that the relationship probably evolved from a parasitism. However, in the field the benefits of the interaction usually outweigh the costs. This may have helped facilitate the evolution of species-specificity in the *Roridula-Pameridea* system.

3. Spiders decrease the number of hemipterans and hence impact negatively on *Roridula* by indirectly decreasing the amount of insect derived nitrogen available to *Roridula*. Nevertheless, mutualism still seems profitable in the field under the highest spider densities and the lowest hemipteran densities. The invasion of spiders is unlikely to cause mutualism collapse, although they may weaken the selective pressures that favour the evolution of species-specificity. As an alternative hypothesis, spiders may also stabilize the mutualism by maintaining hemipterans at

optimal densities. Distribution data suggests that *Roridula* and *Pameridea* share a long history together in the absence of spiders.

4. Although *Roridula* populations show evidence for bottlenecks, fragmentation and inbreeding, reduced plant populations have had no detectable affect on the genetic structure of *Pameridea*. I suspect that this reflects the better dispersal capabilities of *Pameridea* and that this genus functions as metapopulations.

5. Based on electrophoretic variation, it seems as though gene flow of both *Pameridea* and *Roridula* are restricted and should enable host-race formation to occur at the population or regional level. However, the genetic structures of *Pameridea* and *Roridula* are poorly correlated at some hierarchical levels and this relates to spatial distribution patterns being a better predictor of genotype than local adaptation. Genotypic structure (sub specific level) is largely determined by genetic drift or extrinsic adaptation (adaptation to factors other than host phenotype). *Pamerideas'* genotype is not strongly affected by the genotype of *Roridula* at this level. This was supported by the results of "reciprocal transplant" and host choice experiments, which suggest that *Pameridea* have preference hierarchies acting at the specific level but not the sub-specific level. Phenotypic differences in *Roridula* are not large enough to affect *Pamerideas'* genetic structure at the sub specific level. However plant phenotype is likely to have adaptive significance at the specific level.