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Has both plants and associates; no STRUCTURE analysis but genetic distances

COMPARATIVE POPULATION GENETIC STRUCTURES AND LOCAL ADAPTATION OF TWO MUTUALISTS

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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 the two species of a plant genus (*Roridula gorgonias* and *R. dentata*) and their respective obligately associated hemipteran mutualists (*Pameridea roridulae* and *P. marlothi*) using allozymes. In addition, we determine whether genetic structure is related to differences in host choice by *Pameridea*. 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 large distances isolated the plant populations on which they live. Although genetic distances among plant populations were correlated with genetic distances among hemipteran populations, genetic distances of both plants and hemipterans were better correlated with geographic distance. Because *Roridula* and *Pameridea* have different scales of gene flow, adaptation at the local population level is unlikely. However, the restricted gene flow of both plants and hemipterans could enable adaptation to occur at a regional level. In choice experiments, the hemipteran (*Pameridea*) has a strong preference for its carnivorous host plant (*Roridula*) above unrelated host plants. *Pameridea* also prefers its host species to its closely related sister species. Specialization at the specific level is likely to reinforce cospeciation processes in this mutualism. However, *Pameridea* does not exhibit intraspecific preferences toward plants from their natal populations above plants from isolated, non-natal populations.

Key words.—Cospeciation, gene flow, geographical mosaic theory, host tracking, local adaptation, mutualism, vicariance.

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Local adaptation at fine geographic scales may structure parasite populations into genetically distinct groups (demes), thus influencing rates of speciation and community structure (Mopper 1996; Van Zandt and Mopper 1998). In insect-plant relationships, insects may adapt to local plant phenotypes, which may enhance further evolution (Alstad 1998; Nosil et al. 2002; Scriber 2002), and coevolution may result if local adaptations between insects and plants are reciprocal. The geographic scale at which local adaptation and coevolutionary interactions are likely to occur may be strongly affected by the geographical distribution of interacting organisms and their relative abilities to traverse gene-flow barriers (Thompson and Cunningham 2002). Interacting organisms often have incongruent patterns of genetic structure (e.g. Althoff and Thompson 1999), suggesting that patterns of gene flow are not necessarily similar among these organisms. The relative gene flow of hosts and parasites has been shown to potentially affect the coevolutionary outcomes of the interaction (Gandon et al. 1996; Gandon 2002), and this is likely to be true of mutualisms as well. Genetic studies on gene-flow patterns should thus be combined with experimental studies on local adaptation (Kaltz and Shykoff 1998; Lively 1999). Local adaptation of parasites may be evident in the form of fitness hierarchies among various parasites on local hosts, whereas local adaptation of hosts may be evident in the form of fitness hierarchies among various hosts on local parasites (Gandon et al. 1996). Ideally, one would need to perform reciprocal translocations, to study parasites (respectively hosts) on natal and non-natal hosts (respectively parasites). However, preference hierarchies of parasites for different hosts may also suggest local adaptation. Such preference hierarchies where parasites prefer their natal host populations above non-natal host populations have been shown for, e.g., Lepidoptera (Singer et al. 1988; Thompson 1988).

Some studies have examined local adaptation in the context of plant-insect interactions by using experimental methods (e.g. Singer et al. 1988; Thompson 1988; Strauss 1990; Hanks and Denno 1994; Mopper and Simberloff 1995; Bossart 1998; Downie 1999; Mopper et al. 2000). A few have done so in conjunction with studies on genetic structure and gene flow (de Jong et al. 2001; Nice et al. 2002; Funk et al. 2002; also see Feder et al. 1988, 1990; Roderick 1996; Leebens-Mack et al. 1998; Peterson and Denno 1998; Caillaud 1999) although these studies only examined the genetic structure of the parasites and not the hosts. However, to properly understand the evolution of closely associated organisms, it is necessary to examine the population genetic structures of both organisms involved (Thompson 1994; Dybdahl and Lively 1996). Recent studies have compared the population genetic structure of interacting species (Mulvey et al. 1991; Michalakis et al. 1994; Dybdahl and Lively 1996; Davies et al. 1999; Delmotte at al. 1999; Martinez et al. 1999; Jerome and Ford 2002a,b; Mutikainen and Koskela 2002), although these

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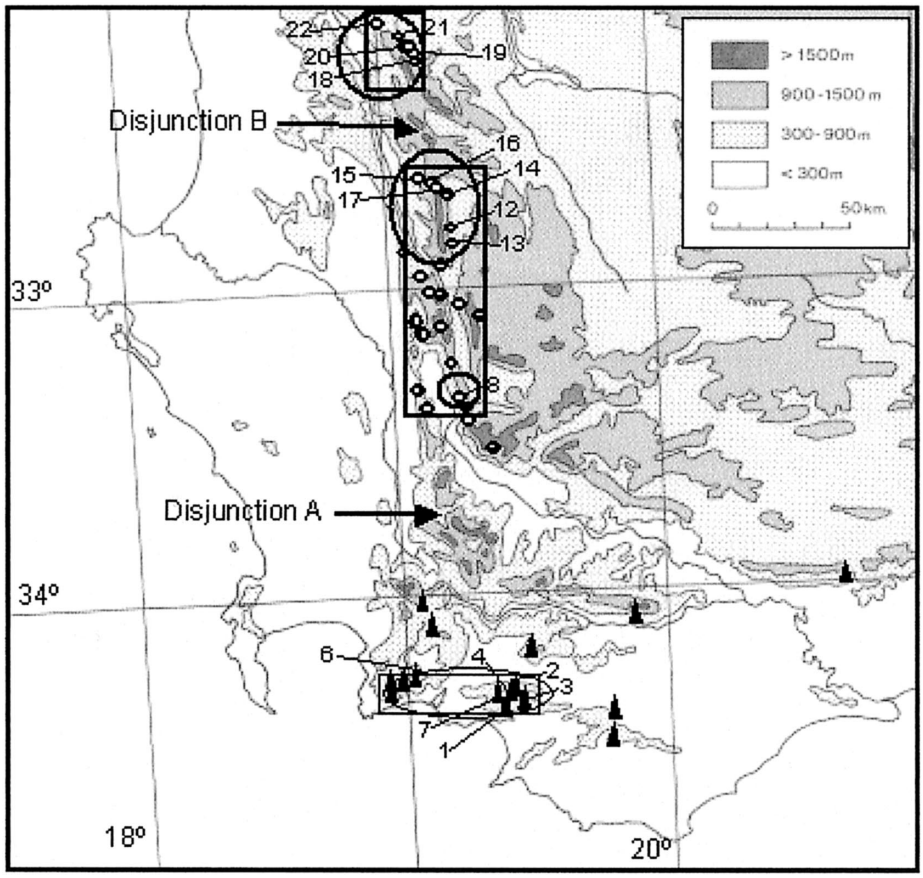

FIG. 1. Map of the south western Cape (South Africa), showing known *Roridula* and *Pameridea* populations (they are exactly the same). Circles represent *R. dentata-P. marlothi* populations and triangles represent *R. gorgonias-P. roridulae* populations. Populations that are numbered were used in the genetic analysis. Two significant gaps in the distribution range of *Roridula* are labeled ''disjunction A'' and ''disjunction B.'' Major neighbor-joining clades are mapped as circles (*Roridula*) and squares (*Pameridea*; see Results and Fig. 3).

studies lack an experimental component to examine local adaptation. Until now only one of these studies (Michalakis et al. 1994) has investigated and correlated the genetic structures of both an insect and its host plant, and a single study has considered mutualistic organisms (Parker and Spoerke 1998, on *Amphicarpa* and *Rhizobium*). Further, a geographical perspective as used by Althoff and Thompson (1999) is crucial in understanding these interactions better and few studies use this approach.

In mutualistic systems, correlated patterns of genetic structure might facilitate coevolution and possibly cospeciation. However, in contrast to host-parasite interactions, selection on mutualisms is expected to be conservative (for further arguments about the different expectations compared to antagonistic systems, in particular the suggestion that mutualistic systems are expected to evolve more slowly than antagonistic ones see Bergstrom and Lachmann 2003). Thus although correlated patterns of gene flow might allow divergent mutualisms to further evolve, phenotypic divergence of interaction traits such as host preference is not necessarily expected to occur even when facilitated by restricted gene flow of both partners of an interaction (however, for reasons why arms races might actually occur among mutualists, see Pellmyr and Huth 1994; Pellmyr 2003).

In this paper, we address some of the gaps in the fields of comparative genetic structures, local adaptation, and speciation by studying a species-specific, insect-plant mutualism. Using allozyme polymorphisms, we examine the population genetic structures of an insect and its host plant from a geographical perspective and then conduct host preference experiments to determine whether local adaptation (as measured here by preference) corresponds to genetic structuring.

The host plant in this study is the carnivorous (Anderson and Midgley 2003) plant family Roridulaceae with one genus and two species, *Roridula dentata* and *R. gorgonias*. Both species are found in allopatric, discrete, patchy populations (see Fig. 1). Distribution records of *R. dentata* suggest a large disjunction between the central and northern parts of its range. *Roridula* is perennial and typically inhabits nutrient poor habitats (Obermeyer 1970); 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 specialist hemipteran, *Pameridea marlothi* on *Roridula dentata* and *P. roridulae* on *R. gorgonias* (Dolling and Palmer 1991). Adults lay their eggs

in the woody tissue of *Roridula* plants. Eggs take about one month to hatch and instars take about one month to reach maturity. Adults live for about 45 days. They seem unable to catch insects by themselves. Both juveniles and adults eat insect prey. Hemipterans also suck plant sap and juveniles eat pollen as well. Although adults are occasionally found on flowers, normally one only finds juveniles on the flowers. Flowers of *Roridula* are self-compatible and the stigma is receptive before anthers ripen although there is overlap, which facilitates self-pollination. Plants are pollinated primarily by the juveniles *Pameridea*, which are flightless and hence do not move much from plant to plant (Anderson et al. 2003). Adult *Pameridea* are capable of flying, although in windless conditions they do not seem to be able to fly more than 2 m at a time. *Pameridea* are never observed on any plants other than *Roridula*. Very little is known about seed dispersal mechanisms in *Roridula*. Seeds fall from a dehiscent capsule and they do not seem to move very far (populations are generally very discrete and occupy areas of small size). There seem to be no adaptations to wind or insect dispersal. As a result, gene flow between plant populations is predicted to be low. For seeds to germinate, burning is necessary. In plant populations that have been recently burnt, hemipterans may be absent for the short period necessary for them to recolonize. It could be that nutrients are abundant soon after fire and hence the hemipterans are not necessary for this short time. As a result, gene flow between hemipteran populations is predicted to be somewhat larger than between plant populations, and strongly influenced by the geographic distribution and burning patterns of the plants.

We examined the genetic structure of the two obligate mutualistic hemipterans and their host plants. Because populations are discrete and isolated, it is expected that significant genetic differentiation among populations may be observed for both hemipterans and plants. Because the gene flow in plants is expected to be low, we predict that plant populations are possibly phenotypically different, enabling local adaptation to occur. We examine population genetic structures from a geographical perspective to determine at what scales adaptation to host phenotype is most likely to occur. 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 both plants and insects. To determine whether genetic structuring is related to tracking of intrinsic traits, we performed host preference experiments on natal versus non-natal populations of host plant and also on closely related species. If insects are locally adapted to particular host types, then we expect them to prefer and perform better on their own host populations/species in comparison to other host populations (Singer et al. 1988).

Materials and Methods

Genetic Structure

Collection and electrophoresis

To determine the distributions of *Roridula* species, collection data was recorded from the Bolus herbarium (University of Cape Town, South Africa) and Compton herbarium (Kirstenbosch Botanical Gardens, Cape Town, South Africa). In addition, new localities were sampled after conservation officials, farmers and biologists provided distribution records. Between 1999 and 2001, all correctly recorded localities were visited and we sampled both *Pameridea* and *Roridula* from as many populations as we could find. Several populations were not relocated, either due to recent local extinctions (mostly due to fire) or incomplete locality data. Seed material was collected from 11 *R. dentata* localities (Pop8, Pop12, Pop14, Pop15, Pop16, Pop17, Pop18, Pop19, Pop20, Pop21, Pop22; see Fig. 1) and six *R. gorgonias* localities (Pop1, Pop2, Pop3, Pop4, Pop6, Pop7). *Pameridea* were collected from nine *R. dentata* localities (Pop8, Pop12, Pop13, Pop14, Pop15, Pop16, Pop18, Pop20, Pop22) and five *R. gorgonias* localities (Pop1, Pop2, Pop3, Pop6, Pop7). The discrepancy between the numbers of hemipteran and plant sites sampled is mostly due to a paucity of hemipterans at some sites. Seeds were not collected from the Pop13 site 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 (one per plant) along two perpendicular, bisecting transects from within half a meter of the transect line. The 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.

Leaf material of *Roridula* is laden with tannins and hence it is difficult to resolve allozymes from leaves. We were able to obtain interpretable banding patterns only from seed material. Seed material (including endosperm) is routinely used in plant allozyme studies (e.g. Harder and Barrett 1995). During fertilization, one male gamete fuses with two female polar bodies to form the triploid endosperm. However, the two polar bodies are the products of a mitosis event and so they have exactly the same genotype. It is thus impossible to get a triallelic seed, even though endosperm is triploid. Hence it is possible to analyze all material using programs designed for diploid individuals.

Seeds placed on ice and live hemipterans were taken back to the laboratory where they were both frozen at $-80°C$. Whole hemipterans were homogenized 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 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 (Whatman no. 3, Whatman Plc., Kent, U.K.) were dipped into the supernant of centrifuged samples and these were inserted into 13% horizontal starch gels. The sample tissue of the whole insects 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. For the plants, care was taken not to use more than one seed per capsule for each locus investigated.

In *Roridula*, the enzymes MDH (Enzyme Commission [EC]

no. 1.1.1.37), ADH (EC 1.1.1.1), GPI (EC 5.3.1.9), and DIA (EC 1.6.2.2) were resolved on a continuous Histidine-citrate buffer system, pH 6.0 (Stuber et al. 1977). Pep LGG (EC 3.4.11.4), IDH (EC 1.1.1.42), ME (EC 1.1.1.40), and PGM (EC 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-PDH (EC 1.1.1.49) and MPI (EC 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 (EC 2.7.4.3), ME, and G6-PDH were also resolved on a continuous Tris-borate-EDTA buffer system at pH 8.6 (Markert and Faulhaber 1965). The enzyme SOD (EC 1.15.1.1) was also resolved while staining for MPI on the above buffer system. The enzymes PGD (EC 1.1.1.44), MDH, ACN (EC 4.2.1.2), 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 (EC 3.1.1.1), GPI, HEX (EC 2.7.1.1), and ARK (EC 2.7.3.3) for *Pameridea*.

Statistical analysis

Single-locus analyses of local mating system.—For each locus and within each sample, departure from random union of gametes was tested by a score test (*U* test), with the alternative hypothesis of heterozygotes deficiency. The tests were performed using Genepop, version 3.4 (Raymond and Rousset 1995). In a given sample, the test could not be performed for monomorphic loci or whenever only two alleles occurred and one of them occurred as a single copy. Global tests across loci or across samples were performed using the multisample score test of Rousset and Raymond (1995). This test assumes the independence of loci. Single-locus Wright's F_{IS} (Wright 1951) were estimated by the estimator $\hat{f}$ of Weir and Cockerham (1984). Multilocus estimates were computed in a slightly different manner than in Weir and Cockerham (1984) or in Weir (1996), in a way that gives more weight to the estimate of more intensively typed loci, in proportion to the intensity of typing (see Rousset 2001, and Genepop, ver. 3.4).

Two-locus analyses of linkage disequilibria within population.—Tests for the independence of genotypes across loci (genotypic linkage disequilibrium) were performed (Fisher's exact tests) for all pairs of loci within each sample. Unbiased exact *P*-value estimates were obtained by the Markov chain method computed by Genepop version 3.4 (Raymond and Rousset 1995). The sequential Bonferroni-type correction (Rice 1989) was applied whenever necessary to correct for significance in multiple tests, in particular when testing panmixia and linkage equilibrium.

Single-locus analyses of population differentiation.—Differentiation over all samples and over all sample pairs was tested using probability tests (or Fisher's exact tests). Unbiased estimates of the associated *P*-values were calculated using the Markov chain method computed by Genepop version 3.4 (Raymond and Rousset 1995). Polymorphic loci were used to estimate single-locus Wright's *F*-statistics F_{ST} (Wright 1951) by the estimator $\hat{\theta}$ of Weir and Cockerham (1984). Recent studies (for a review see e.g., Bohonak 1999) have suggested that the pairwise F_{ST} among populations is a good predictor of the amount of effective gene flow between them. This has been questioned by Whitlock and McCauley (1999), because F_{ST} reflects actual gene flow only under various assumptions unlikely to be met in most data sets. However, in general there is a quantitative agreement between relative F_{ST} values and what is known about actual dispersal in particular species (Hamrick and Godt 1996; Bohonak 1999). We focused on relative values of F_{ST} (of plants compared to insects, within regions compared to among regions, etc.), thus the issues rightly raised by Whitlock and McCauley (1999) were not of great concern.

To relate the dispersal capabilities of *Roridula* and *Pameridea* to geographic distributions we plotted multilocus estimates of F_{ST} between all pairwise populations with the corresponding pairwise geographic distances. Isolation by distance was analyzed by computing the regression of pairwise F_{ST} estimates between pairs of populations to their geographical distance. Correlations were made using the Mantel permutation procedure (Mantel 1967) associated with the Spearman rank correlation coefficient as test statistics using Genepop (Raymond and Rousset 1995). We also repeated the analysis after excluding the single geographically outlying population (Pop8) to determine whether 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 because we had some F_{ST} values of one, rendering the above calculation meaningless. For both within- and between-population analyses, sequential Bonferroni correction was applied to test for significance whenever multiple tests were performed (Rice 1989).

Analyses of genetic distances.—Pairwise genetic distances between pairs of populations were computed using Cavalli-Sforza's chord measure (Cavalli-Sforza and Edwards 1967), obtained from the Gendist program (Phylip, ver. 3.6; Felsenstein 1989, 2002). The distance trees were reconstructed using the neighbor-joining method of Saitou and Nei (1987) using the Neighbor program of Phylip. The robustness of each node was evaluated by bootstrapping data over individuals, for 1000 replications, using the Seqboot program (Phylip, ver. 3.6). The resulting consensus tree was obtained through the program Consense (Phylip, ver. 3.6) and displayed with the program Treeview (Page 1996).

Hierarchical analyses.—In addition, because *R. dentata* and *P. marlothi* have a wide distribution, genetic differentiation in these species were also analyzed at a regional level. Populations were broadly categorized as belonging to one of three regions, namely North, Central, and South (see Figs. 1 and 2). A hierarchical analysis of variance (Weir and Cockerham 1984) allowed us to decompose the covariance of allelic frequencies into its various components: between genes within individuals, among individuals within populations, among populations within regions, and among regions. For this analysis we used Arlequin version 2 (Schneider et al. 2000), which also allowed us to estimate Wright's *F* statistics (F_{IS} within population, F_{PR} among populations within regions, and F_{RT} among regions) according to Weir and Cock-

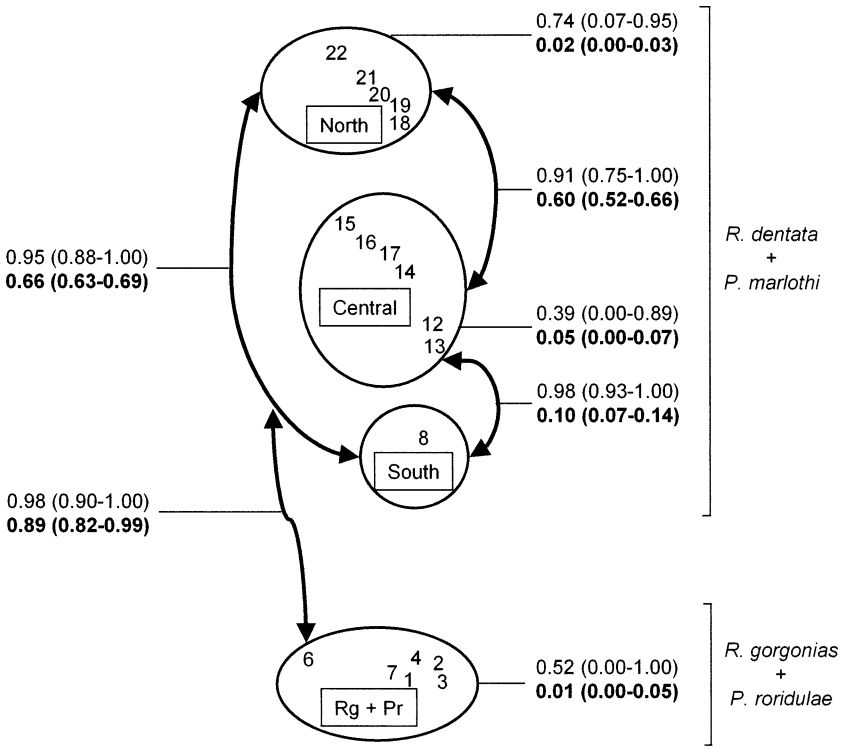

FIG. 2. Average paired F_{ST} values (range) for *Roridula* and *Pameridea* (*Pameridea* values are bold). One species pair (*R. dentata* and *P. marlothi*) has been divided into three geographical regions (North, Central, and South). The other species pairing of *R. gorgonias* and *P. roridulae* (Rg + Pr) has a narrow distribution and hence is not divided into regions. We measured population pairwise F_{ST} within regions, between regions, and between species.

erham (1984). Permutation tests implemented in Arlequin were used to test for significance of the various covariance components and associated *F*-statistics.

Host Preference

Parasite preference for different host plants has sometimes been found to be 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. We examined preference in *Pameridea* to determine whether it preferred its natal host species or host population above others. *Pameridea* have never been observed on any plants other than *Roridula*, and this genus has no closely related families in the fynbos (Dolling and Palmer 1991; B. Anderson, pers. obs.). Other carnivorous plants (*Drosera* sp.) were too small to be reasonable control plants. Thus, cuttings of the genus *Leucadendron* (a common, noncarnivorous Proteaceae) were used as controls in the choice experiments because this genus represents one of the dominant families in the matrix that *Pameridea* have to cross to recolonize new populations. 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* cuttings, and *P. roridulae* were all 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 *Roridula*'s range ensuring that *R. gorgonias* and *R. dentata* are geographically separated by about 70 km (Fig. 1). *Roridula dentata*, *Leucadendron*, and *P. marlothi* 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 (see Results).

Equal numbers of males and females of each *Pameridae* species were made to choose between a cutting from their natal plant population and from a cutting of similar proportions of the other host plant species. Choices were also made between cuttings of the original host-plant species and *Leucadendron* cuttings (both from their natal plant population), as well as a plastic plant. These last choice tests were used to indicate the maximal level of preference that might be expected. 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 nonhost) 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 shin-

ing 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 (this might have introduced a bias in our experiments toward individuals with the best ability to recognize their host). 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. roridulae* were tested in this way for each possible pairing of *R. gorgonias* natal population with *R. dentata*, *Leucadendron*, or a plastic plant. Similarly, 40 *P. marlothi* were made to choose between *R. dentata* from their natal population, *R. gorgonias*, *Leucadendron*, and plastic plants (except that, because the test for preference was marginally significant with $n = 40$, 80 insects were tested for the choice between *R. dentata* and *R. gorgonias*). In addition, 40 *P. marlothi* from the northern population were made to choose between *R. dentata* from the natal population and *R. dentata* from the central population. Reciprocally, 40 *P. marlothi* from the central population were also made to choose between *R. dentata* from their natal population and *R. dentata* from the northern population. Choices were tested using a chi-squared test with one degree of freedom.

Results

Population Genetic Structure

Roridula

Locus polymorphism.—Eight of the nine loci examined in the six *R. gorgonias* populations were monomorphic (Table 1A). Only the 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).

Five of the 12 loci examined in the 11 *R. dentata* populations were monomorphic (Table 1A). A maximum of four alleles were detected per polymorphic locus. Most local populations were fixed. 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, 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).

Hardy-Weinberg equilibrium.—Of the 10 cases of polymorphism (two in *R. gorgonias* and eight in *R. dentata*), no locus was found to be in Hardy-Weinberg equilibrium (*U* test, $P < 0.05$) prior to Bonferroni correction. Only two loci showed no significant deficiency of heterozygotes when adjusting *P*-values for multiple testing (locus MDH-1 in Pop3 of *R. gorgonias*, and PGM-2 in Pop22 of *R. dentata*). Multiple sample tests for heterozygote deficiency in *R. dentata* were significant for each population across all polymorphic loci and each locus across all polymorphic populations. In both *R. dentata* and *R. gorgonias*, the estimates of F_{IS} were very large ($F_{IS} = 0.97$ and 0.93, respectively), suggesting a strongly inbred mating system.

Two-locus linkage disequilibrium within populations.—No test could be performed in *R. gorgonias* as a single locus was polymorphic. In *R. dentata*, none of the three tests that could be performed (all in Pop19) showed significant linkage disequilibrium, even though such disequilibria would be expected in a population with an inbred mating system (as is the case of Pop19, results not shown). The testing power is likely to be very low because of the low level of polymorphism.

Differentiation among populations.—Genetic differentiation was very high at all levels investigated (Fig. 2). The seven fixed allele differences (with no allele in common) suggest a complete lack of effective gene flow between species. 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. 2). All F_{ST} population pairings between *Roridula* species were highly significant ($P < 0.0001$).

At the intraspecific level, in *R. dentata*, overall differentiation was very large ($F_{ST} = 0.90$, $P = 0$; *P*-value provided by Fisher's method for overall differentiation). There were fixed differences between regions of *R. dentata*. As a result, the average paired F_{ST} value between different regions was high (0.88–0.95 depending on the region) and the range in F_{ST} was small (Fig. 2; see Table 2A for significance levels of individual population pairings). All pairings between regions were significantly different. Populations that were separated by distances greater than 40 km had F_{ST} values very close to one. Thus the pairwise F_{ST} between the southern and central *R. dentata* populations were consistently large ($F_{ST} = 0.93$–1.00) with relatively little variation, suggesting complete isolation between these regions (Fig. 2). Similarly, high F_{ST} values were observed between northern and both southern and central populations ($F_{ST} = 0.75$–1.00 and 0.88–1.00 respectively).

Pairwise F_{ST} values were highly variable within regions (Table 2A and Fig. 2; $F_{ST} = 0.00$–0.95) but most population pairs within regions were significantly different after Bonferroni sequential correction of unbiased estimates of the *P*-value of the probability tests (or Fisher' exact tests) provided by Genepop (ver. 3.4; Table 2A; note, however, that Pop12, Pop14, and Pop17 were all monomorphic for the same alleles). Those paired F_{ST} values that 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 0.43 ($P < 0.0001$; Table 2A). This suggests very little or no recurrent gene flow within regions, and similarity among some populations is more likely due to a relatively recent common origin (historical gene flow).

For *R. gorgonias*, overall differentiation was also very large at the single polymorphic locus ($F_{ST} = 0.76$) and highly significant ($P = 0$). Similar to the F_{ST} pairings within regions for *R. dentata*, pairwise F_{ST} values were highly variable within the distribution range sampled ($F_{ST} = 0.00$–1.00; Table

TABLE 1. *Roridula* and *Pameridea* allele frequencies. Positive frequencies are given in bold. NA, not applicable (missing data).

A. *Roridula*

							Roridula dentata											
	Roridula gorgonias						South	Central					North					
Locus	Pop1	Pop2	Pop3	Pop4	Pop6	Pop7	Pop8	Pop12	Pop14	Pop15	Pop16	Pop17	Pop18	Pop19	Pop20	Pop21	Pop22	
LGG-1																		
N	13	13	10	17	13	14	5	18	4	18	22	16	23	16	30	5	21	
A	0	0	0	0	0	0	0	**1**	**1**	**1**	**1**	**1**	0	0	0	0	0	
B	**1**	**1**	**1**	**1**	**1**	**1**	0	0	0	0	0	0	0	0	0	0	0	
C	0	0	0	0	0	0	**1**	0	0	0	0	0	**1**	**1**	**1**	**1**	**1**	
GPI-1																		
N	13	13	10	16	12	15	5	18	4	18	21	17	23	16	31	5	20	
A	0	0	0	0	0	0	0	0	0	0	0	0	**0.870**	0	0	0	0	
B	0	0	0	0	0	0	0	**1**	**1**	**1**	**1**	**1**	0	0	0	0	0	
C	0	0	0	0	0	0	**1**	0	0	0	0	0	**0.130**	**0.188**	**1**	**1**	0	
D	**1**	**1**	**1**	**1**	**1**	**1**	0	0	0	0	0	0	0	0	0	0	0	
E	0	0	0	0	0	0	0	0	0	0	0	0	0	**0.812**	0	0	**1**	
ADH-1																		
N	13	13	10	16	13	15	5	18	4	18	22	17	20	16	35	5	20	
A	0	0	0	0	0	0	**1**	0	0	0	0	0	0	0	0	0	0	
B	**1**	**1**	**1**	**1**	**1**	**1**	0	0	0	0	0	0	0	0	0	0	0	
C	0	0	0	0	0	0	0	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	
DIA-1																		
N	13	13	10	16	13	15	5	18	4	18	22	16	21	16	30	5	20	
A	0	0	0	0	0	0	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**0.875**	**0.933**	**1**	**1**	
B	0	0	0	0	0	0	0	0	0	0	0	0	0	**0.125**	**0.067**	0	0	
C	**1**	**1**	**1**	**1**	**1**	**1**	0	0	0	0	0	0	0	0	0	0	0	
PGM-2																		
N	17	16	16	16	14	16	5	20	6	21	19	19	19	10	24	5	16	
A	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**1**	0	
B	**1**	**1**	**1**	**1**	**1**	**1**	0	0	0	0	0	0	0	0	0	0	0	
C	0	0	0	0	0	0	0	**1**	**1**	**1**	**1**	**1**	**1**	**0.950**	**1**	0	**0.938**	
D	0	0	0	0	0	0	**1**	0	0	0	0	0	0	**0.050**	0	0	**0.062**	
G6-PDH-1																		
N	10	17	11	11	11	11	5	11	6	17	15	18	17	10	10	5	11	
A	**1**	**1**	**1**	**1**	**1**	**1**	0	0	0	0	0	0	0	0	0	0	0	
B	0	0	0	0	0	0	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	
MDH-1																		
N	18	22	16	16	14	16	6	21	6	22	20	19	18	9	24	5	16	
A	0	0	0	0	0	0	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	
B	**1**	**0.523**	**0.062**	**0**	**1**	**1**	0	0	0	0	0	0	0	0	0	0	0	
C	0	**0.477**	**0.938**	**1**	0	0	0	0	0	0	0	0	0	0	0	0	0	
MPI-1																		
N	0	0	0	0	0	0	6	17	3	20	15	18	17	9	15	5	14	
A	NA	NA	NA	NA	NA	NA	**1**	0	0	0	0	0	0	0	0	0	0	
B	NA	NA	NA	NA	NA	NA	0	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	
IDH-1																		
N	0	0	0	0	0	0	6	18	6	19	19	20	16	9	22	5	13	
A	NA	NA	NA	NA	NA	NA	0	0	0	**0.895**	**0.368**	0	0	0	0	0	0	
B	NA	NA	NA	NA	NA	NA	**1**	**1**	**1**	**0.105**	**0.632**	**1**	**1**	**1**	**1**	**1**	**1**	
LGG-2																		
N	13	13	10	17	13	17	5	18	4	18	22	16	23	6	30	5	21	
A	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	
ME-1																		
N	17	16	16	16	14	16	6	20	6	21	19	19	19	6	24	5	16	
A	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	
IDH-2																		
N	0	0	0	0	0	0	6	18	6	19	19	20	16	16	22	5	13	
A	NA	NA	NA	NA	NA	NA	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	

TABLE 1. Continued.

B. *Pameridea*

Locus	*Pameridea roridulae*					*Pameridea marlothi*								
						South	Central					North		
	Pop1	Pop2	Pop3	Pop6	Pop7	Pop8	Pop12	Pop13	Pop14	Pop15	Pop16	Pop18	Pop20	Pop22
MPI-1														
N	30	24	30	20	19	20	20	20	15	20	20	21	20	25
A	0	0	0	0	0	**0.075**	0	0	**0.167**	0	0	**0.048**	**0.025**	**0.120**
B	**1**	**1**	**1**	**1**	**1**	**0.925**	**0.925**	**1**	**0.833**	**0.925**	**1**	**0.952**	**0.975**	**0.880**
C	0	0	0	0	0	0	**0.075**	0	0	**0.075**	0	0	0	0
PGD-1														
N	20	20	13	20	14	20	15	9	16	20	15	15	22	8
A	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**0.867**	**0.818**	**0.875**
B	0	0	0	0	0	0	0	0	0	0	0	**0.133**	**0.182**	**0.125**
PGM-2														
N	24	20	32	24	20	20	19	20	15	20	30	21	20	32
A	0	**0.025**	0	**0.042**	0	0	0	0	0	0	0	0	0	0
B	0	0	0	0	0	**0.050**	**0.026**	**0.025**	0	0	0	0	0	0
C	0	0	0	0	0	**0.950**	**0.974**	**0.975**	**1**	**1**	**1**	**0.024**	0	0
D	**0.125**	**0.050**	0	**0.125**	**0.025**	0	0	0	0	0	0	**0.976**	**1**	**1**
E	**0.875**	**0.925**	**1**	**0.833**	**0.950**	0	0	0	0	0	0	0	0	0
F	0	0	0	0	**0.025**	0	0	0	0	0	0	0	0	0
ME-1														
N	15	35	10	20	19	40	25	20	15	20	40	25	30	23
A	**1**	**1**	**1**	**1**	**1**	0	0	0	0	0	0	0	0	0
B	0	0	0	0	0	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**
MDH-1														
N	20	15	30	30	25	40	20	10	15	25	25	28	33	28
A	**1**	**1**	**1**	**1**	**1**	0	0	0	0	0	0	0	0	0
B	0	0	0	0	0	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**
ACN-1														
N	26	31	15	25	37	20	19	19	15	33	20	15	23	23
A	0	0	0	0	0	**0.225**	**0.053**	**0.000**	0	**0.045**	0	0	**0.065**	0
B	**0.962**	**1**	**1**	**1**	**1**	**0.775**	**0.710**	**0.842**	**1**	**0.758**	**0.950**	**1**	**0.935**	**1**
C	**0.038**	0	0	0	0	0	**0.237**	**0.158**	0	**0.197**	**0.050**	0	0	0
AK-1														
N	15	30	20	15	15	20	19	10	17	15	20	45	35	30
A	**1**	**1**	**1**	**1**	**1**	0	0	0	0	0	0	0	0	0
B	0	0	0	0	0	**0.975**	**0.842**	**0.950**	**0.706**	**0.633**	**0.925**	**0.159**	0	0
C	0	0	0	0	0	**0.025**	**0.158**	**0.050**	**0.294**	**0.367**	**0.075**	**0.841**	**1**	**1**
DIA-1														
N	30	18	15	40	39	0	0	0	0	0	0	0	0	0
A	0	0	0	**0.063**	0	NA	NA	NA	NA	NA	NA	NA	NA	NA
B	**0.950**	**0.833**	**1**	**0.900**	**0.872**	NA	NA	NA	NA	NA	NA	NA	NA	NA
C	**0.050**	**0.167**	0	**0.037**	**0.128**	NA	NA	NA	NA	NA	NA	NA	NA	NA
EST-1														
N	36	34	40	23	15	20	15	15	14	19	15	21	20	20
A	0	0	0	0	0	0	**0.067**	0	0	**0.079**	0	0	0	0
B	0	0	0	0	0	**0.050**	**0.067**	**0.033**	**0.214**	**0.079**	**0.067**	**0.047**	**0.050**	**0.175**
C	**0.042**	**0.088**	0	**0.022**	0	**0.950**	**0.800**	**0.700**	**0.786**	**0.816**	**0.800**	**0.667**	**0.750**	**0.525**
D	**0.903**	**0.868**	**1**	**0.956**	**0.900**	0	0	**0.167**	0	**0.026**	0	**0.286**	**0.175**	**0.300**
E	**0.055**	**0.044**	0	**0.022**	**0.100**	0	**0.066**	**0.100**	0	0	**0.133**	0	**0.025**	0
G6PDH-1														
N	37	24	35	20	13	15	19	16	27	19	20	21	22	25
A	**0.054**	**0.021**	0	**0.050**	0	0	0	0	0	0	0	0	0	0
B	0	0	0	0	0	**0.133**	**0.053**	0	**0.019**	**0.026**	**0.100**	**0.048**	**0.068**	0
C	0	0	0	0	0	**0.867**	**0.947**	**1**	**0.981**	**0.974**	**0.900**	**0.952**	**0.932**	**1**
D	**0.946**	**0.979**	**1**	**0.950**	**1**	0	0	0	0	0	0	0	0	0

TABLE 1. Continued.

B. *Pameridea*

Locus	Pameridea roridulae					*Pameridea marlothi*								
						South	Central					North		
	Pop1	Pop2	Pop3	Pop6	Pop7	Pop8	Pop12	Pop13	Pop14	Pop15	Pop16	Pop18	Pop20	Pop22
GPI														
N	40	36	31	41	20	20	20	25	27	20	35	35	34	40
A	0	0	0	0	0	0	0	0	0	0	0	**0.014**	**0.015**	**0.038**
B	0	0	0	0	0	**0.975**	**0.950**	**1**	**1**	**0.950**	**0.914**	0	0	0
C	0	0	0	0	0	**0.025**	**0.050**	0	0	**0.050**	**0.086**	**0.986**	**0.985**	**0.962**
D	**0.063**	**0.041**	0	0	**0.100**	0	0	0	0	0	0	0	0	0
E	**0.887**	**0.903**	**1**	**0.915**	**0.900**	0	0	0	0	0	0	0	0	0
F	**0.050**	**0.028**	0	**0.085**	0	0	0	0	0	0	0	0	0	0
G	0	**0.028**	0	0	0	0	0	0	0	0	0	0	0	0
HEX														
N	23	20	35	20	20	20	20	20	17	26	35	15	20	15
A	0	0	0	0	0	0	**0.025**	0	0	**0.038**	0	0	0	0
B	0	0	0	0	0	**1**	**0.975**	**1**	**1**	**0.962**	**1**	**1**	**1**	**1**
C	**1**	**1**	**1**	**1**	**1**	0	0	0	0	0	0	0	0	0
IDH-1														
N	21	20	14	24	36	20	19	19	15	31	24	14	21	19
A	**0.310**	**0.175**	**0.321**	**0.208**	**0.264**	0	**0.026**	0	**0.033**	0	0	0	0	0
B	**0.500**	**0.500**	**0.572**	**0.604**	**0.625**	**0.825**	**0.526**	**0.658**	**0.467**	**0.484**	**0.417**	**0.643**	**0.714**	**0.526**
C	**0.190**	**0.325**	**0.107**	**0.188**	**0.111**	**0.150**	**0.395**	**0.316**	**0.467**	**0.435**	**0.479**	**0.286**	**0.214**	**0.448**
D	0	0	0	0	0	**0.025**	**0.053**	**0.026**	**0.033**	**0.081**	**0.104**	**0.071**	**0.072**	**0.026**
IDH-2														
N	21	20	14	24	36	20	19	19	15	32	24	15	22	22
A	0	0	0	0	0	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**
B	**1**	**1**	**1**	**1**	**1**	0	0	0	0	0	0	0	0	0
SOD														
N	23	20	35	20	20	20	20	20	17	26	35	15	20	15
A	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**
ARK														
N	40	36	31	41	20	20	20	25	27	20	35	35	34	40
A	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**

2B, Fig. 2). In particular, populations Pop1, Pop6, and Pop7 were fixed for the same allele at all loci, including at locus MDH-1, which was polymorphic in Pop2 and Pop3. For this reason we set F_{ST} among these populations to zero. F_{ST} was very low (F_{ST} = 0.0001) for the pair of Pop3 and Pop4, which are 0.5 km apart. However, other nearby *R. gorgonias* populations had high, significant F_{ST} values.

Genetic distances and geographical patterns.—The genetic distance-based tree of the 17 *Roridula* populations reveals two distinct groups, supported by a 100% bootstrap value, not surprisingly corresponding to the two species (Fig. 3A). Within the cluster formed by *R. dentata* populations, three geographically distinct subclusters are clearly discernable (Fig. 2): the single southern population separated from the other *R. dentata* populations with a 61% bootstrap value, and the northern populations separated from other populations in 92% of the cases. Hierarchical analyses, isolation-by-distance patterns, and associated statistical tests are referred to in the last part of Results.

Pameridea

Locus polymorphism.—Nine of the 16 loci resolved for *P. roridula*e 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 (Table 1B). A sixth locus was almost monomorphic because 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 (Table 1B). For example, fixed differences were observed for the locus PGM-2 and GPI, distinguishing the northern populations from all others. Strong frequency differences were also found for the locus AK, which also corresponded to a difference between northern versus central and southern populations.

Fixed differences between the two *Pameridea* species were observed for seven loci (47%) (ME-1, MDH-1, AK-1, G6–1, GPI, HEX, and IDH-2). Of the remaining loci, allele frequency differences ranged from large (PGM-2 and EST-1) to small (MPI, PGD-1, ACN-1, and IDH-1) to none at all (SOD and ARK).

Hardy-Weinberg equilibrium.—Of the 72 cases of polymorphic loci within populations for which equilibrium could

TABLE 2. Pairwise F_{ST} estimates and significance of differentiation (below diagonal) and geographic distance (kms; above diagonal) organized by regions for (A) *Roridula dentata*, (B) *R. gorgonias*, (C) *Pameridea marlothi*, and (D) *P. roridulae*. Differentiation was tested using a probability test (see Material and Methods): ns, $P > 0.05$; * $0.05 > P > 0.001$; ** $0.001 > P$ (within each species, *P*-values estimated by Genepop ver. 3.4 were adjusted by sequential Bonferroni procedure). F_{ST} among Pop12, Pop14, and Pop17 in *R. dentata* and F_{ST} among Pop1, Pop6, and Pop7 in *R. gorgonias* have been arbitrarily set to zero (see text). Note that F_{ST} estimates involving identical populations are not necessarily equal because of unequal sample sizes (see Materials and Methods).

A.

	South	Central					North				
	Pop8	Pop12	Pop14	Pop15	Pop16	Pop17	Pop18	Pop19	Pop20	Pop21	Pop22
Pop8		59	76	74	74	74	116	116	117	134	138
Pop12	1**		24	22	23	23	63	63	63	63	80
Pop14	1**	0.00		4	3	3	46	46	46	46	58
Pop15	0.97**	0.89**	0.83**		0.5	0.9	52	52	52	52	60
Pop16	0.93**	0.33**	0.20**	0.43**		0.3	52	52	52	52	60
Pop17	1**	0.00	0.00	0.90**	0.34**		52	52	52	52	60
Pop18	0.95**	0.93**	0.90**	0.92**	0.84**	0.93**		2	2	11	14
Pop19	0.88**	0.86**	0.77**	0.85**	0.75**	0.86**	0.63**		2	7.5	13
Pop20	0.97**	0.96**	0.94**	0.94**	0.88**	0.96**	0.81**	0.68**		7	11
Pop21	1**	1**	1**	0.96**	0.89**	1**	0.90**	0.77**	0.90**		4
Pop22	0.98**	0.97**	0.96**	0.94**	0.86**	0.97**	0.83**	0.07*	0.89**	0.96**	

B.

	Pop1	Pop2	Pop3	Pop4	Pop6	Pop7
Pop1		4	4	3.5	19	2
Pop2	0.43**		0.3	0.8	21	5
Pop3	0.94**	0.35**		0.5	21	5
Pop4	1**	0.46**	0.00 ns		20.5	4.5
Pop6	0.00	0.39**	0.93**	1**		18
Pop7	0.00	0.41**	0.93**	1**	0.00	

C.

	South	Central					North		
	Pop8	Pop12	Pop13	Pop14	Pop15	Pop16	Pop18	Pop20	Pop22
Pop8		59	54	76	74	74	121	122	135
Pop12	0 07*		4	24	22	23	63	63	80
Pop13	0.07*	0.01 ns		26	24	25	67	67	82
Pop14	0.15**	0.04 ns	0.07*		4	3	46	46	58
Pop15	0.12**	0.00 ns	0.06 ns	0.02 ns		0.5	52	52	60
Pop16	0.11**	0.02 ns	0.04 ns	0.04*	0.05 ns		52	52	60
Pop18	0.63**	0.55**	0.60**	0.58**	0.52**	0.59**		2	14
Pop20	0.68**	0.60**	0.66**	0.63**	0.57**	0.64**	0.00 ns		11
Pop22	0.69**	0.60**	0.66**	0.61**	0.57**	0.64**	0.01 ns	0.04 ns	

D.

	Pop1	Pop2	Pop3	Pop6	Pop7
Pop1		4	4	19	2
Pop2	0.00 ns		0.3	21	5
Pop3	0.01 ns	0.05 ns		21	5
Pop6	0.01 ns	0.01 ns	0.02*		18
Pop7	0.01 ns	0.02 ns	0.02 ns	0.01*	

be tested by an exact test, only two (2.8%) were found to be out of Hardy-Weinberg (HW) equilibrium, consistent with a Type 1 error rate of 0.05. After a Bonferroni correction for multiple comparisons, no departure from local panmixia was observed. A multiple sample score test for heterozygote deficiency over all populations and all loci confirmed local panmixia in *P. marlothi* ($P = 0.09$) but not in *P. roridulae* ($P = 0.02$). However, this was due to a single locus in a single population (IDH-1 in Pop7). Other populations did not show a significant deficiency of heterozygotes for this particular locus and no other locus showed a significant deficiency of heterozygotes for Pop7. The more conservative global test of departure from Hardy-Weinberg using Fisher's method gave no significant departure in either species ($P = 0.43$ in *P. marlothi* and $P = 0.09$ in *P. roridulae*). Accordingly, in both *P. marlothi* and *P. roridulae*, overall F_{IS} were small, especially when compared to plant populations ($F_{IS} = 0.07$ and 0.12, respectively).

Two-locus linkage disequilibrium within populations.—In *P. marlothi*, linkage equilibrium was rejected for a single pair of loci in a single population (G6-PDH and IDH-1 in Pop8) after adjusting *P*-values for multiple testing (127 tests could be performed). Of 41 tests across all populations, this single pair of loci was not in linkage equilibrium either, and no other test was significant. In *P. roridulae*, linkage disequilibrium was never rejected for any of the 43 tests that could be performed per locus and population, and for any of the 19 tests that could be performed across all populations.

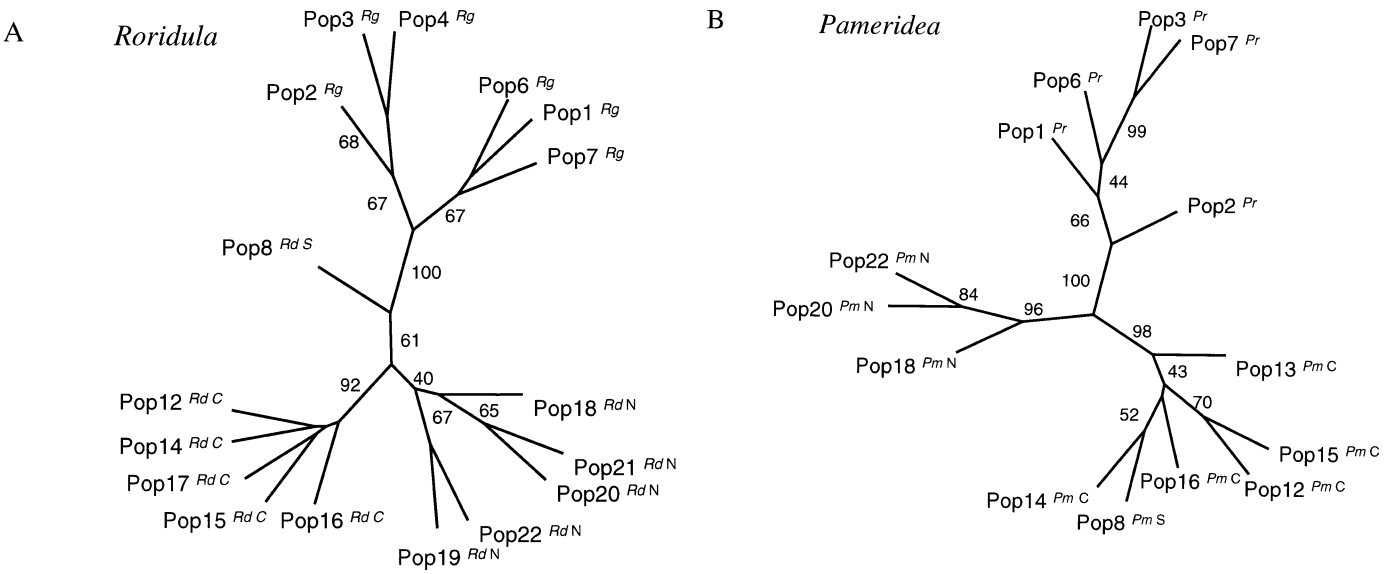

FIG. 3. Neighbor-joining phylogram based on Cavalli-Sforza (1967) chord distance for *Roridula* (A) and *Pameridea* (B) populations. *Rg* and *Rd* denote *R. gorgonias* and *R. dentata* populations; *Pr* and *Pm* denote *P. roridulae* and *P. marlothi* populations. S, C, and N denote *R. dentata* and *P. marlothi* populations at the southern, central, and northern parts of their range. Numbers at nodes indicate the percent of bootstrap samples with such nodes. Only values larger than 40% are shown.

Differentiation among populations.—Several fixed allele differences between the two *Pameridea* species suggest a complete lack of effective gene flow and this is also reflected by the high paired F_{ST} values (F_{ST} = 0.82–0.99) between the two species (Fig. 2). At the intraspecific level, in *P. marlothi*, overall differentiation was large (F_{ST} = 0.51, P = 0; P-value provided by Fisher's method for overall differentiation). Pairwise F_{ST} values were low within regions (Table 2C, Fig. 2; F_{ST} = 0.00–0.07) and most population pairs within regions were not significantly different after Bonferroni sequential correction of unbiased estimates of the P-value of the probability tests (or Fisher's exact tests) provided by Genepop (ver. 3.4) (apart from Pop14 with Pop13 or Pop16; Table 2C). This suggests that a large amount of effective gene flow consistently occurs within regions. In contrast, all pairings between regions were significantly different. However, the pairwise F_{ST} between the southern and central *R. dentata* populations were comparatively low (F_{ST} = 0.07–0.15) with relatively little variation, suggesting consistently good effective gene flow between these regions (Fig. 2). In contrast, high F_{ST} values were observed between northern and both southern and central populations (F_{ST} = 0.63–0.69 and 0.57–0.66 respectively).

For *P. roridulae*, overall differentiation was low (F_{ST} = 0.01) although it was highly significant (P = 0). Pairwise F_{ST} values were very low within the distribution range sampled (F_{ST} = 0.00–0.05) and only two of them (Pop6 with Pop3 and Pop7) in 10 pairs corresponded to significant differentiation after adjusting P-values using sequential Bonferroni procedure (Table 2D, Fig. 2).

Genetic distances and geographical patterns.—The genetic distances-based tree (Fig. 3B) of the 14 *Pameridea* populations also reveals two main groups, corresponding to the two species. These two clusters were separated with a bootstrap value of 100%. The cluster corresponding to *P. marlothi* can further be separated into two distinct clusters, one grouping the northern populations, the other the central populations as well as the unique southern one (bootstrap value of 98% see Figs. 1 and 3B).

Hierarchical analysis of gene frequencies in Roridula dentata and Pameridea marlothi

Table 3 shows the covariance components of allele frequencies as well as Wright's F-statistics estimated using Arlequin software (Schneider et al. 2000) according to Weir and Cockerham (1984). In the plant *R. dentata*, all F-statistics are significant, but most of the variance occurs among populations within regions (49% of total variance), and to a lesser extent among regions (34%). Conversely, in the insect *P. marlothi*, the only significant differentiation occurs among regions, which corresponds to 51% of the total variance of allele frequencies. Most of the remaining variance occurs among individuals within populations. The exclusion of Pop8, the lone southern population, reduces the among-regions component of variance in the plant to 62% of its original value (Table 3; compare 0.14 and 0.09), suggesting that this population does contribute to this overall component, whereas its exclusion increases this component by 15% in the insect (Table 3; compare 0.15 and 0.18), as expected from Figure 3, where Pop8 clearly clusters within the central region.

Correlating genetic structures of Roridula and Pameridea

In *R. gorgonias*, there was no significant correlation between pairwise F_{ST} and geographic distances (r = 0.03, P = 0.30; Mantel test). There was no significant correlation between *P. roridulae* F_{ST} and geographic distances (r = −0.52, P = 0.92). Finally, no significant correlation was found be-

TABLE 3. Hierarchical analysis of covariance of allele frequencies in *Roridula dentata* and *Pameridea marlothi*: *F*-statistics and covariance components (σ). Percentages in parentheses indicate the proportion of each covariance component to the total variance in allele frequency. ns, $P > 0.05$; * $0.05 > P > 0.01$; ** $0.01 > P > 0.001$; *** $0.001 > P > 0.0001$.

	F_{IS}	F_{IT}	F_{PR} (among populations, within regions)	F_{RT} (among regions)	σ_G (among genes within individuals)	σ_I (among individuals within populations)	σ_S (among populations within regions)	σ_P (among regions)
Roridula dentata (Pop8 included)	0.96***	0.99***	0.75***	0.34**	0.00195 (0.66%)	0.04682 (15.90%)	0.14463 (49.13%)	0.14463 (34.31%)
Roridula dentata (Pop8 excluded)	0.96***	0.99***	0.74***	0.31**	0.00206 (0.72%)	0.04943 (17.34%)	0.14451 (50.69%)	0.08909 (31.25%)
Pameridea marlothi (Pop8 included)	−0.65 ns	0.20 ns	0.01 ns	0.51*	0.23604 (80.02%)	−0.09315 (−31.58%)	0.00107 (0.36%)	0.15101 (51.20%)
Pameridea marlothi (Pop8 excluded)	−0.61 ns	0.25 ns	0.01 ns	0.53*	0.24855 (74.64%)	−0.09398 (−28.22%)	0.00094 (0.28%)	0.17748 (53.30%)

tween the pairwise F_{ST} of *P. roridulae* and the corresponding F_{ST} of *R. gorgonias* ($r = 0.38$, $P = 0.10$).

In contrast, *R. dentata* populations had positive and significant correlations between pairwise F_{ST} and geographic distances ($r = 0.51$, $P < 0.01$; Fig. 4A). Positive and significant correlation of pairwise F_{ST} versus geographic distances was also found for the hemipterans *P. marlothi* that live on *R. dentata* ($r = 0.71$, $P < 0.01$; Fig. 4B). The correlation seems mostly due to the high F_{ST} values for population pairings involving the northern populations (Fig. 4B). The correlation of *R. dentata* and *P. marlothi* F_{ST} was also significant; however, the correlation coefficient was lower than for correlations between pairwise F_{ST} and geographic distances ($r = 0.48$, $P < 0.01$; Fig. 4C). Dropping the outlying Pop8 did not affect the broad outcome of the analyses but did increase correlation coefficients for tests involving *P. marlothi* while *P*-values remained low ($r = 0.92$ instead of 0.71 for isolation by distance, and $r = 0.60$ instead of 0.48 for *P. marlothi* vs. *R. dentata*, $P < 0.01$ for both).

Host Preference

All results are summarized Figure 5. *Pameridea roridulae* significantly preferred their host plant, *R. gorgonias*, above *R. dentata* (29:11, $\chi^2 = 8.1$, $P < 0.01$), the plastic plant (36:4 $\chi^2 = 25.6$, $P < 0.001$), and the *Leucadendron* control (33:7, $\chi^2 = 16.9$, $P < 0.001$ (Fig. 5A). Similarly *P. marlothi* also displayed preferences toward their species *R. dentata* above all other choices (53:27, $\chi^2 = 56.9$, $P < 0.001$ for *R. gorgonias*; 32:8, $\chi^2 = 14.4$, $P < 0.001$ for the plastic plant; and 31:9, $\chi^2 = 12.1$, $P < 0.001$ for *Leucadendron*; Fig. 5B). However, when faced with the choice of *R. dentata* from their natal population versus nonnatal *R. dentata*, neither *P. marlothi* from northern nor from the central populations chose their natal plants preferentially (northern population: 21 cases of choice of own *R. dentata* plant population vs. 19 cases of choice of a population from the central region, $\chi^2 = 0.1$, $P = 0.75$; central population: 23 cases of choice of own plant population vs. 17 cases of choice of a population from the northern region, $\chi^2 = 0.9$, $P = 0.34$).

DISCUSSION

Population Genetic Structure

Roridula

Roridula populations are strongly subdivided at all levels, although a large variance occurs for the amount of pairwise differentiation among population pairs. Both species of *Roridula* are characterized by strong deficits of heterozygotes in all populations with polymorphic loci. Selfing due to lack of pollen dispersal is 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. 2003). Inbreeding is likely to promote the genetic and phenotypic divergence of *Roridula* populations. Subdivisions based on pairwise F_{ST} values show strong divisions not only between species but also between close intraspecific populations confined to small regions. These populations could possibly exhibit phenotypic differences to which *Pameridea* could adapt at local or regional scales.

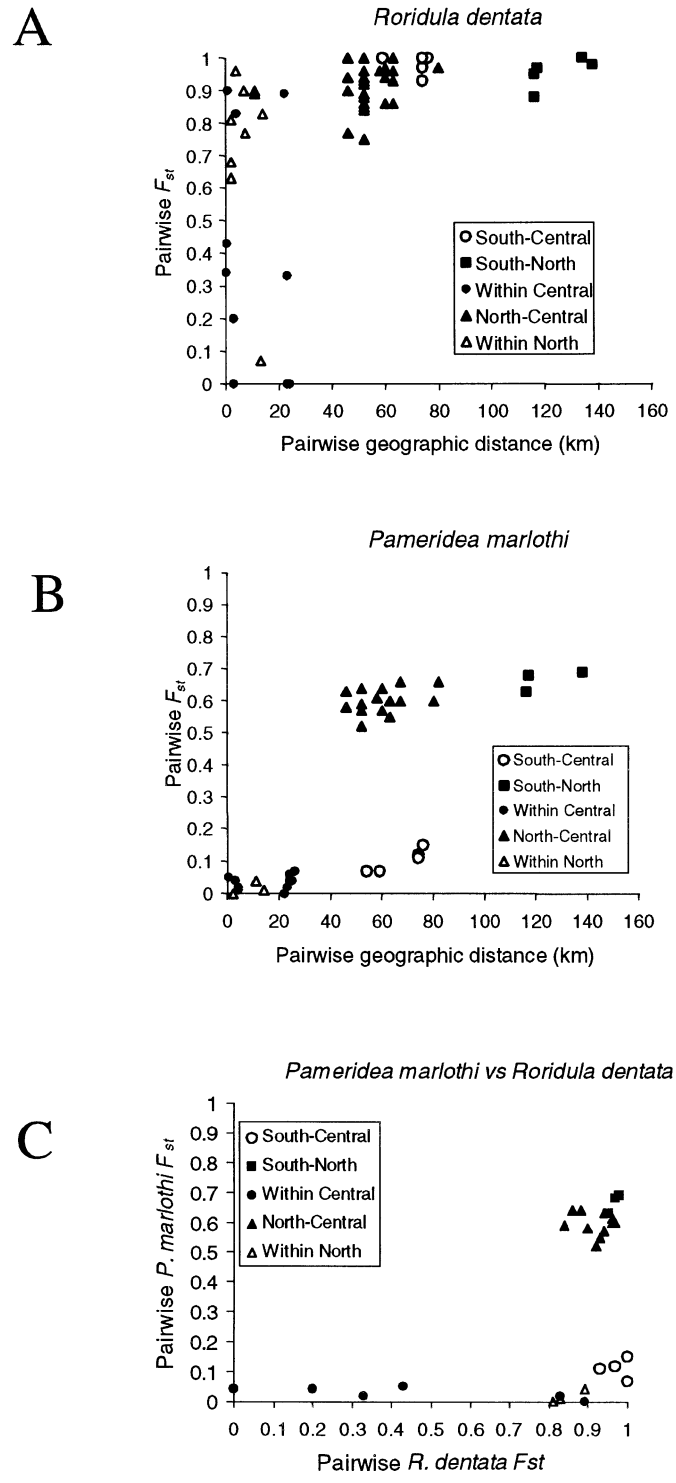

FIG. 4. Isolation-by-distance patterns and correlated structures using F_{ST} as a measure of genetic differentiation. (A) *Roridula dentata*. (B) *Pameridea roridulae*. (C) Plant (*R. dentata*) versus hemipteran (*P. marlothi*) F_{ST}.

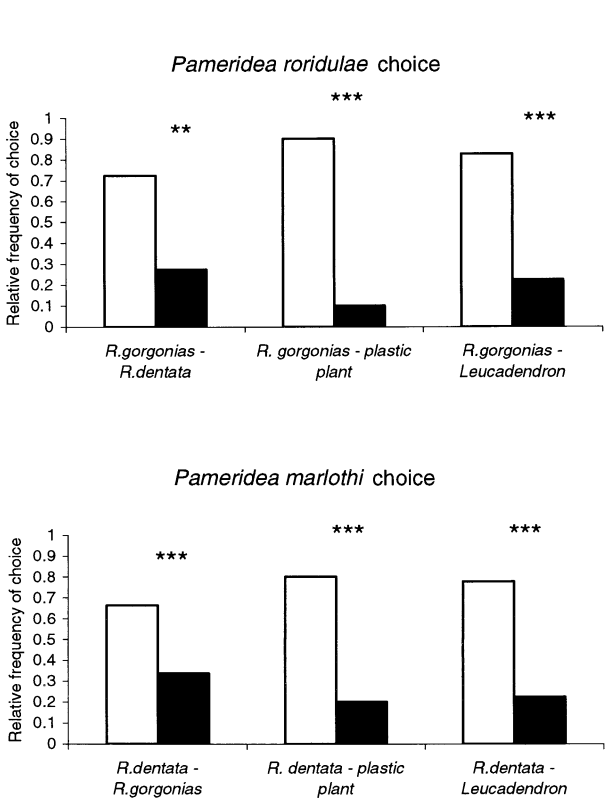

FIG. 5. The relative frequency of choices made by *Pameridea* (A, *P. roridulae*; B, *P. marlothi*) for either the natal host plant species (A, *Roridula gorgonias*; B, *R. dentata*) or nonhost species. Choices for the host plant are clear bars and choices for the nonhost plant are dark bars. Statistical significance (χ^2 test) is indicated by asterisks: ** $0.01 > P > 0.001$, *** $P < 0.001$. Sample size is 40 in each test except for the choice of *P. marlothi* between *R. dentata* and *R. gorgonias* ($n = 80$).

The high F_{ST} values for *Roridula*, including for some very close by populations, suggest that even limited effective gene flow between most populations is rare or nonexistent. As a result, the genetic patterns in this genus are probably an artifact of ancient or historical events. In support of this, De Meester et al. (2002) hypothesized that high genetic differences between nearby populations often reflect 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. However, there are 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 effective gene flow cannot be ruled out. Although only a single polymorphic locus was resolved for *R. gorgonias*, nevertheless the species 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

As with *Roridula* plants, the division of *Pameridea* into two separated, distinct species based on morphological char-

acters (Dolling and Palmer 1991) was confirmed by allozymes. Fixed allele differences at several loci clearly indicate no effective gene flow and thus reproductive isolation. However, because 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 effective gene flow in the hemipterans due to their better dispersal capabilities. A very strong gene flow barrier exists that separates the northern hemipteran populations from the others. The distribution of allelic variation among the loci examined is consistent with other incipient insect species (Thorpe and Sole-Cava 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 intermediate populations to facilitate hemipteran movement and gene flow. Northern populations are highly isolated (Fig. 1) with no 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 effective gene flow is relatively high. The most plausible explanation for this is that several populations between these regions (Fig. 1) act as intermediates for effective 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, consistent with normal observations of insects where the range in F_{ST} usually varies from 0.01 to 0.20 (McCauley and Eanes 1987; Roderick 1996; Peterson and Denno 1998).

Correlating genetic structures of Roridula and Pameridea

There are no patterns of isolation by distance in *R. gorgonias*. It could be that statistical power is too low for showing such pattern, as a single locus was polymorphic, whereas several polymorphic loci were found in *R. dentata*, which also has a much larger sampled range size. Such a low amount of polymorphism in *R. gorgonias* could reflect a recent bottleneck in this species. In addition, only six *R. gorgonias* populations were analyzed. Although several polymorphic loci were resolved for *P. roridulae*, only five populations were analyzed. In this species, no significant differentiation was found between populations that were all less than 30 km apart. Hence, no isolation by distance was found in either *P. roridulae* or *R. gorgonias*. There was also no correspondence between the pairwise F_{ST} of these two species, which may (or may not) also be related to the paucity of polymorphic loci for *R. gorgonias*.

In contrast, isolation-by-distance trends were observed in both *R. dentata* and *P. marlothi*, but the geographic scale at which gene flow was limited by geographical distance differed between the two organisms. As a result of the very low effective gene flow among local populations within a region in *R. dentata*, its present disjunct distribution pattern is unlikely to influence the future genetic structure in this species. In contrast, effective gene flow in *P. marlothi* populations seems strongly affected by its contemporary distribution patterns, as we discussed in the previous section. Hence there is a discrepancy between the genetic structures of *P. marlothi* and *R. dentata* (Fig. 4C) that results in comparatively poor (albeit significant) correlations of their pairwise F_{ST}. Since strong effective gene flow seems to exist between populations of *P. marlothi* within a region but not between isolated regions, it may be expected that local adaptation would most likely occur at a regional level, especially if regions are geographically isolated (Law and Koptur 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. Nevertheless, the fact that there is a significant correlation between genetic structures of these two mutualists may encourage local adaptation to take place.

Our study suggests that cospeciation has taken place in the *Pameridea-Roridula* complex and that cospeciation may be in progress in the northern *R. dentata* and *P. marlothi* populations. Furthermore, allozyme results suggest that local adaptation at some level can potentially play a role in structuring mutualist populations, since the swamping effects of gene flow on local adaptation are likely to be minimized by the patchy host distribution and poor hemipteran dispersal across large discontinuities. Despite this, regional distribution patterns and different dispersal attributes most strongly influence the genetic structures of *R. dentata* and *P. marlothi*. Plants and hemipterans have clearly different scales of effective 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), in contrast to our system, showed that a fungal pathogen (*Microbotryum*) was more genetically structured than its plant host (*Silene*). However, as we did, they attributed this difference to the fact that the pathogen self-fertilizes and has low effective gene flow, whereas the host-plant is an outcrosser with good effective 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,b) found the opposite.

Because *Pameridea* and *Roridula* have such different scales of effective gene flow, *Pameridea* are unlikely to adapt to plant phenotype at the plant population level, because there would be too much gene flow from individuals facing different plant phenotypes of other populations. Only in isolated populations (as in the extreme north) are interregional gene flow patterns between plants and hemipterans more congru-

ent. Such groups of isolated populations may represent evolutionary hotspots because hemipterans may be able to adapt to plants at the regional level. These findings are in accordance 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 (see also Nuismer et al. 1999; Gomulkiewicz et al. 2000). 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. 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. However, the ability of gene flow to swamp local adaptation depends on both the rate of effective gene flow and the strength of selection. Because *R. dentata* host populations are highly differentiated, they might generate strong enough selection pressures for local adaptation in *Pameridea*. If heritable variation for fitness exists in *P. marlothi* then local adaptation may result even in the face of relatively high gene flow. Moreover, gene flow may also, just as mutations, bring evolutionary novelties upon which natural selection can act (e.g., for a model see Gandon et al. 1996), so that gene flow is not necessarily antagonistic of local adaptation (for further discussion on the neglected role of gene flow and hybridization as a creative role for the spread of advantageous mutations see Rieseberg and Carney 1998; Rieseberg and Burke 2001).

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 subspecific level. For example, Mulvey et al. (1991) examined the genetic structure of deer and parasitic helminths. In this study, there was no correlation between genetic differentiation and geographic 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. However, they did not 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 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 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,b). 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. One possible mechanism explaining this quite surprising observation could be that the host and the parasite migrate together, moreover in a way unrelated to geographic distance. A similar pattern of co-structure might also be expected (but here we would need more than verbal models) if selective interactions between hosts and parasites are strong enough that linkage disequilibria build up between genes for local adaptation and neutral markers. Jerome and Ford (2002a,b) 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 or migration rate. Patterns of genetic structure based on putatively neutral markers can elucidate long-term average rates of gene flow and genetic drift. Comparisons of genetic structure between populations of interacting species provide insights into how these processes may be influencing coadaptation and codiversification. Conversely, it could be that neutral genetic structures are themselves influenced by the process of coadaptation itself, in a way that still needs to be modeled. 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.

Host Preference

The present study suggests that despite fine-scale genetic structure of hosts and parasites, there is no evidence for local specialization, even at the regional scale. However, we did find interspecific specialization suggesting that host tracking may occur once hosts have significantly diverged. 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, noncarnivorous 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. We attribute this to strong (obvious) differences in plant phenotype although this study does not distinguish between visual or olfactory differences. Differences in plant morphology (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* and *P. roridulae* 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 northern and central regions did not preferentially choose plants from their populations of origin. This may be because the experimental procedure is not fine-tuned enough to pick up such subtle differences. Alternatively differences in plant morphology might not be large enough at this taxonomic level to elicit adaptive responses in *Pameridea*. Indeed, such morphological differences could not be found between populations of *R. dentata*, whereas there are striking differences between the two *Roridula* species (B. Anderson, pers. obs.). As pointed out earlier in this paper, in a mutu-

alistic system there might be no inherent tendency to select for divergence, which could explain why the host preference toward the natal population has apparently not evolved in this system. Alternatively, it could be that allozyme polymorphism evolves faster than host preference.

Our results are very similar to those of Reed and Hafner (1997) who found no local adaptation at subspecific 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 morphology are only great enough to cause divergence in lice once gopher populations have already diverged significantly (i.e., speciated). We 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. Before completely ruling out local or regional adaptation in this system, it is necessary to perform reciprocal translocation experiments to examine the fitness of *Pameridea* on natal versus non-natal *Roridula* populations and of *P. marlothi* on *R. gorgonias* and reciprocally.

Conclusions

This study of *Roridula-Pameridea* differs from previous analyses of host-parasite genetic structure in that most of those dealt with antagonistic relationships whereas this study examines a mutualism. Moreover, the present study also examines genetic structure from a geographical perspective and includes experimental data on host preference. Our study adds to the small body of literature on host-parasite genetic structures by showing that similar questions arise 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 effective 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. However, preliminary data presented here using host choice suggest no evidence for local adaptation at the regional scale. Instead, evidence supports adaptation at the specific level.

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