20 Frugivore-generated Seed Shadows: a Landscape View of Demographic and Genetic Effects

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Introduction

A seed shadow is the spatial pattern of seed distribution relative to parent trees and other conspecifics; it results from the process of seed dispersal and represents the starting template for plant regeneration. Janzen (1970) and Connell (1971) consider it the population recruitment surface. For animal-dispersed, endozoochorous species the seed shadow results primarily from movement patterns of frugivores. Presumably, frugivores can dramatically affect both the demography and genetic make-up of animal-dispersed plant species. These effects, however, have rarely been documented in an integrated way.

In this chapter we focus on how frugivores influence the number and spatial pattern of propagules that reach the soil, and their simultaneous influence on gene flow via seed dispersal. We advocate an integrated view of both demographic and genetic effects to understand the role of frugivores on plant recruitment (Alvarez-Buylla *et al.*, 1996). Given that multiple influences sequentially alter after this initial effect of frugivores (i.e. post-dispersal seed predation, germination, seedling mortality), we need to quantitatively assess the relative importance of dispersal by frugivores for plant population biology. Seed dispersal by frugivores is the link in the demographic transition between the ripe fruit crop on the trees and, after delivery, the subsequent stages of establishment of germinated seeds, seedlings, saplings and established adults, i.e. the whole recruitment cycle. Thus, seed dispersal may play a pivotal role in the demography of plant populations (Harper, 1977) by simultaneously influencing not only the numerical dynamics of recruitment from dispersal to establishment, but also the genetic make-up of the seed shadow.

The difficulty of tracking the origin of frugivore-dispersed seeds has precluded a robust analysis of vertebrate seed dispersal (Levey and Sargent, 2000). Indeed, the difficulties in measuring and analysing the dispersal of seeds in natural communities has been considered an unavoidable limitation of the field (Wheelwright and Orians, 1982). Recent developments in molecular biology (Carvalho, 1998), however, have resulted in a series of molecular tools based on DNA analysis that allow analysis of gene-flow patterns via seed dispersal (Ouborg *et al.*, 1999) and the statistical analysis of the resulting patterns of genetic structure (Schnabel *et al.*, 1998a; Luikart and England, 1999).

More specifically, for animal-dispersed species, gene flow via seeds can be estimated

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directly (see Ouborg *et al.*, 1999, for a review). Yet recent studies of gene flow in plants are primarily focused on pollen flow (Sork *et al.*, 1999); very few studies have used molecular markers to assess seed-dispersal patterns. Even fewer studies have linked genetic patterns in fleshy-fruited plant species with the behaviour of frugivores (Loiselle *et al.*, 1995a,b; Schnabel *et al.*, 1998b). Ideally, one should link detailed observations of bird foraging behaviour and movement to distinct types of landscape patches or microhabitats (Wenny and Levey, 1998; Jordano and Schupp, 2000), with monitoring of seed rain (i.e. using seed traps) (Kollmann and Goetze, 1997) and analysis of the genetic make-up of the seeds.

Frugivores thus have the potential to influence both plant demography and genetic structure. This influence, in turn, has an impact on two major arenas of seed dispersal ecology, namely seed-dispersal limitation and landscape patterns of gene flow via seeds.

Demographic effects: seed-dispersal limitation

Dissemination limitation is probably the major demographic effect that frugivore activity can have on plant populations. It occurs whenever seed delivery by frugivores is insufficient to saturate available microhabitats for establishment; in species that are dispersal-limited, increasing seed input would result in increased recruitment (Ehrlén and Eriksson, 2000; Jordano and Schupp, in prep.; see also Schupp *et al.*, this volume; Table 20.1). If we include delayed consequences of frugivore activity for plant recruitment (i.e. the quantity and quality components of disperser effectiveness) (Schupp, 1993; Jordano and Schupp, 2000), three major forms of limitation processes may operate through the dispersal stage of plant regeneration (Table 20.1; Jordano and Schupp, in prep.; see also Schupp *et al.*, this volume): seed source limitation, dissemination limitation and limitation of establishment. We now review these briefly.

A demographic limitation operating during the seed-dispersal and establishment stages simply represents a low realized recruitment relative to the maximum potential. Thus, a primary form of dissemination limitation arises whenever the seed crop is insufficient to reach all the available safe sites ('source limitation' *sensu* Clark *et al.* (1999a)). This type of limitation is clearly independent of frugivore activity; it simply results from low fruit production.

Table 20.1. Demographic effects of frugivores through limitation of seed dispersal. Frugivore activity can limit plant population recruitment by both direct influences on fruit-removal levels and seed-delivery patterns and indirect (delayed) effects via the influences on the survival prospects of dispersed seeds and established seedlings. A non-restrictive view of seed-dispersal limitation would have to include the multiple effects of frugivores at all the stages of plant recruitment (Jordano and Schupp, 2001; Schupp *et al.*, this volume).

Stage and limitation	Definition
1. Seed limitation	Seed production at population level is insufficient to saturate all available safe sites (source limitation, *sensu* Clark *et al.*, 1999a)
2. Dissemination limitation	
2.1. Quantitatively restricted seed dispersal	Independent of the quantity of seeds produced, disperser activity is insufficient to disperse all seeds away from parents
2.2. Distance-restricted seed dispersal	Independent of the quantity of seeds dispersed away from parents, most seed dispersal is short-distance
2.3. Spatially contagious seed dispersal	Independent of distance of seed dispersal, seeds are not spread evenly, but rather are deposited patchily (aggregated), with many seeds in some sites and few to none in most sites
3. Establishment limitation	Independent of number of seeds arriving in a site, biotic and abiotic factors limit establishment of new individuals (i.e. delayed consequences in respect of Stage 2)

A second aspect of seed-dispersal limitation, dissemination limitation, derives from the role of frugivores in seed dissemination (Table 20.1). It includes all processes associated with frugivore foraging that limit the number, distance and/or spatial distribution of seeds over the landscape. But, independently of the quantity of seeds successfully dispersed away from parent trees, dispersal can be limited by distance-restricted seed delivery (e.g. due to territory defence) (Snow and Snow, 1988) and/or spatially aggregated patterns of seed delivery (e.g. in the vicinity of parent trees or conspecifics, at lek sites, latrines, roosts, etc.) (Dinerstein and Wemmer, 1988; Izhaki *et al.*, 1991; Chapman and Chapman, 1995; Julliot, 1997; Wenny and Levey, 1998). Spatial aggregation is a characteristic feature of animal-generated seed shadows (Fig. 20.1; Harms *et al.*, 2000).

Finally, establishment limitation might occur as a consequence of frugivore activity if, independently of the number of seeds dispersed away from parents, frugivores fail to deliver seeds to the safest sites for germination and establishment (Table 20.1).

Genetic effects: identifying the source tree of dispersed seeds and characterizing the genetic make-up of the seed shadow

Frugivores influence the genetic make-up of the seed shadow, i.e. the particular combination of dispersed genotypes and their location relative to parent trees and other conspecifics. Thus, a sizeable fraction of gene flow in endozoochorous species can be attributed to frugivore activity but, as far as we know, no study has yet dissected the contributions of

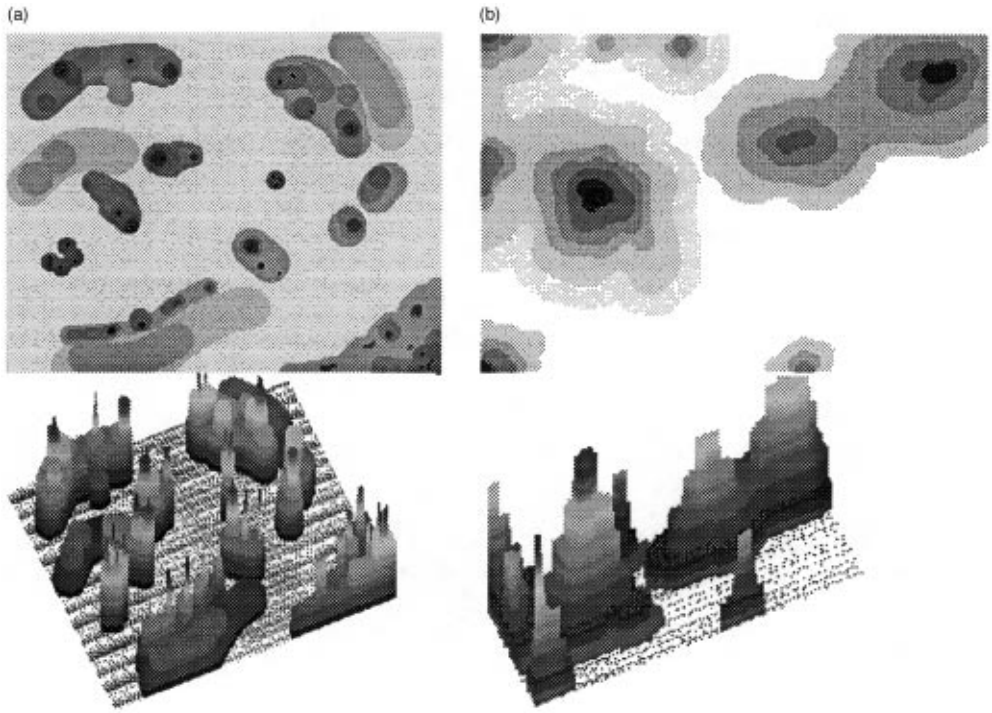

Fig. 20.1. Idealized representation of the landscape pattern of seed shadows in (a) animal-dispersed species (e.g. *P. mahaleb*) (Jordano and Schupp, 2000; J.L. García-Castaño and P. Jordano, unpublished), and (b) abiotically (wind)-dispersed species (e.g. *Acer mono*, *Betula grossa*) (Nakashizuka *et al.*, 1995). Darker shading or higher profile (in the lower panels) indicates greater seed density. Seed densities range from 1 to 140 seeds m^{-2} in (a) and from 1 to 60 seeds m^{-2} (*A. mono*) or 2 to > 5600 seeds m^{-2} (*B. grossa*) in (b).

pollination and seed dispersal to gene flow in animal-dispersed species. Gene flow via pollen is certainly extensive, especially in obligate outcrossed species, such as many woody endozoochorous species (Hamrick and Godt, 1997), but the scanty evidence available suggests that seed dispersal is also important (Dow and Ashley, 1996; Schnabel *et al.*, 1998b). By genotyping animal-dispersed seeds we can resolve several long-standing issues in seed-dispersal ecology, including the spatial relationships (e.g. distance, aggregation) between the maternal tree and its propagules, the full characterization of the seed shadow and the provenance of dispersed seeds. In addition, we can better understand gene-flow patterns in complex landscapes, where habitat heterogeneity may impose dramatic patterns of genetic structure (Cain *et al.*, 2000).

To identify the maternal tree of seeds dispersed by frugivores, we compare the multilocus genotype of the seed endocarp (which is diploid maternal tissue in angiosperm species with drupaceous fruits) (Roth, 1977) with the genotype of adult trees in the population, obtained from leaf tissue (Godoy and Jordano, 2001). Using this procedure, dispersal distances can be estimated for individual seeds defecated or regurgitated by frugivores, sampled in our regular monitoring of seed rain using seed traps scattered in the forest. This represents a major advance in seed dispersal, opening new avenues for research by combining ecological data with molecular tools (Godoy and Jordano, 2001).

Recent applications of hypervariable molecular markers, such as simple sequence repeats (SSR), or microsatellite loci, to seed-dispersal studies (Ouborg *et al.*, 1999) allow inference of a seed's or plant's maternal tree, providing a powerful tool in seed-dispersal ecology to assess dispersal distances directly. Using spatial autocorrelation techniques, a few studies have documented extreme clumping of progeny, either relative to the parents or to related individuals (Sork *et al.*, 1993; Loiselle *et al.*, 1995a; Schnabel *et al.*, 1998b; Streiff *et al.*, 1998; Ueno *et al.*, 2000). Such clumping is probably common in fleshy-fruited species with highly non-random and aggregated seed rain (Clark *et al.*, 1999a; Harms *et al.*, 2000). In most cases, however, the fine-scale patterns of genetic structuring cannot be linked to specific foraging modes or seed-deposition patterns by frugivores, and the role of dispersers is largely inferred.

Our goal in this chapter is to outline a protocol for an integrated analysis of the demographic and genetic effects of frugivores of plant populations. These two types of frugivore effects have rarely been considered together. We examine dispersal limitation and attempt to establish conceptual bridges with genetic effects to allow a more integrated understanding of frugivore effects in plant communities. We introduce molecular methods to unambiguously identify the maternal tree of seeds dispersed by frugivores and discuss the relevance of this approach for understanding the evolutionary effects of frugivores on plant populations. Finally, we discuss potential implications for future research.

Methods

Study site and species

This study was conducted in the Reserva de Navahondona-Guadahornillos (Parque Natural de las Sierras de Cazorla, Segura y las Villas, Jaén province, south-eastern Spain), with the main study site located at Nava de las Correhuelas (1615 m elevation). Deciduous vegetation covers deep soils. Small trees and shrubs are mixed with extensive patches of grassland, while adjacent rocky exposed slopes are dominated by open pine forest (*Pinus nigra*, subsp. *salzmannii*) with juniper. Extensive patches of open habitat (~66% of surface area), with either grassland, gravelly soil or rock outcrops, appear with shrubs and small trees, both isolated and clumped, giving way to open pine forests (Jordano, 1993; Jordano and Schupp, 2000, for detailed description). *Prunus mahaleb* is a small tree (2–10 m height) growing scattered at mid-elevations (1200–2000 m) in south-eastern Spanish mountains. It is relatively abundant at Nava de las Correhuelas, with an estimated population of ~180 reproductive individuals. *Prunus mahaleb* has insect-pollinated flowers; approximately equal proportions of solitary bees and

flies act as pollinators (see Jordano, 1993, for details).

Frugivorous birds visiting *P. mahaleb* trees include legitimate seed-dispersers (warblers, *Sylvia* spp.; robin, *Erithacus rubecula*; thrushes, *Turdus* spp.; and redstarts, *Phoenicurus* spp.), which swallow fruits whole and defecate and/or regurgitate seeds, usually after leaving the tree. Some species, however, peck the fruit, tearing off the pulp and dropping the seed to the ground beneath the parent (e.g. tits, *Parus* spp., and chaffinch, *Fringilla coelebs*) (Jordano and Schupp, 2000, and references therein). Seed rain of *P. mahaleb* in the study area is generated by frugivorous birds and, marginally, by carnivorous mammals (J.L. García-Castaño and P. Jordano, 1999, personal observation). Seed rain is highly patchy, largely restricted to covered microhabitats beneath woody vegetation close to fruiting trees (Jordano and Schupp, 2000).

Frugivorous birds were watched during observation periods at focal trees in the study area. Basic data on feeding rates (i.e. the number of fruits taken per visit, etc.), the microhabitat type of the first perch used after leaving the feeding tree and its distance from the focal tree were recorded for each observation (for details, see Jordano and Schupp, 2000). Nine types of microhabitats were defined according to the presence of shrub cover, height of vegetation and whether or not a rocky substrate was present (Jordano and Schupp, 2000). For the present analyses we pooled some types, resulting into six distinct microhabitats: *P. mahaleb* fruiting trees, low shrubs (*Berberis vulgaris*, *Juniperus communis*), mid-height shrubs (*Crataegus monogyna*, *Lonicera arborea*, etc.), pine trees (including those with and without low shrub cover beneath them) and, as open substrates, deep soil and rocky substrates (including gravelly soil, rocks with soil and rock boulders). By combining the information on number of visits recorded, mean number of seeds dispersed per visit, proportion of exit flights to each microhabitat and seed-rain data, we were able to estimate the contribution of each main disperser species to the seed rain in each microhabitat (for details see Jordano and Schupp, 2000).

Methods: seed-addition experiments

Experimental design

We used a factorial design, with treatments for seed addition, exclusion of post-dispersal seed predators (rodents) and type of microhabitat. Seeds were sown at ten replicate locations in each of four microhabitat types (as defined in Jordano and Schupp, 2000). Two microhabitats had woody plant cover: beneath *P. mahaleb* trees and beneath pine trees with low shrubs (mostly *Juniperus* and *B. vulgaris*, < 1 m height); the other two, gravelly soil and deep soil with grassy cover, were open microhabitats with no shrub or tree cover.

The seed-addition treatment included two levels of seed-sowing density. The 'control' level had seed density adjusted to the median background seed density recorded for each microhabitat type in a concurrent sampling of seed-rain patterns (Jordano and Schupp, 2000). The 'added', or seed-addition, level had sowing density adjusted to the 95% percentile of the seed rain, roughly a threefold increase in the number of seeds sown in the control.

Finally, the predator exclusion treatment consisted of wire-mesh enclosures that were either closed to exclude predators or open to allow them access. Thus, each replicate plot in a given microhabitat type had four subplots for seed sowing, according to the combinations of the seed-addition ('added' and 'control' levels) and rodent-exclusion treatments ('open' and 'excluded' levels).

Methods: genotyping of individual dispersed seeds

Seed sampling

Seeds for genetic analyses were sampled with seed traps (aluminum trays protected with 0.8 cm wire mesh; see Jordano and Schupp, 2000) during the 1996 fruiting season. Sets of two seed traps were located beneath the canopy of each of five 'focal' *P. mahaleb* maternal trees selected as illustrative of the range of tree sizes and growing in sites and neighbourhood densities typical of the species in the study population. In addition, sets of two seed traps

were located away from each focal tree in 2–3 replicate locations per focal tree, including two microhabitat types (as defined in Jordano and Schupp, 2000): beneath mid-height shrubs and beneath pine trees. Mid-height shrubs (e.g. *C. monogyna*, *Rosa canina* and *L. arborea*) were selected in the neighbourhood of the focal trees (within 10 m) or at more distant locations (> 10 m). Another set of two seed traps per focal tree was located beneath pine trees with low shrubs (two replicate locations) and beneath pine trees with open understorey (three replicate locations). The design included 20 replicate sampling locations totalling 38 seed traps, a stratified random sample of 481 sampling points used in a concurrent study of seed rain.

A total of 95 seeds sampled from the seed traps were analysed. Both defecated and regurgitated seeds were included in the sample, with 37 seeds from traps beneath *P. mahaleb* (sampled at random from the total seed sample captured in these traps) and 58 seeds from traps in other microhabitats. Seeds were kept dry and at room temperature until analysis.

The locations of seed traps and all the adult, reproductive trees in the population were mapped and recorded in a geographical information system (GIS) database. Leaf tissue from adult trees in the population (180 trees including ~100% of the potential maternal trees for 1996 progeny) was collected (see Jordano and Godoy, 2000), kept in liquid nitrogen within labelled duplicate cryotubes and stored at –80°C.

DNA extraction and amplification

DNA was extracted from 100–200 mg of fresh leaf tissue, using the rapid miniprep method of Cheung *et al.* (1993). Tissue was homogenized in 320 µl of extraction buffer (200 mM Tris-HCl pH 8.0, 70 mM ethylenediamine tetra-acetic acid (EDTA), 2 M NaCl, 20 mM sodium bisulphite) with an electric drill (560 W; full speed) with attached plastic disposable pestles. After homogenization, 80 µl of 5% sarcosyl was added and the sample was incubated at 65°C for 30 min and centrifuged at 16,000 *g* for 15 min to remove insoluble material. DNA was precipitated by the addition of 90 µl of 10 M ammonium acetate and 200 µl of isopropanol. The mixture was incubated at room temperature for 5 min and centrifuged for 15 min at 16,000 *g*. The resulting pellet was washed with 70% ethanol, dried and resuspended in 100 µl TE buffer.

This DNA, extracted from leaf tissue of all the potential maternal trees in the population, was used to construct a database of multilocus genotypes of adult trees. These genotypes were matched with those obtained from DNA extracted from the seed endocarp tissue, thereby allowing identification of the maternal tree for each seed (Godoy and Jordano, 2001). To extract DNA from the lignified seed endocarps, we used a similar protocol, with the following modifications: tissue was homogenized in 320 µl of extraction buffer and resuspended in 50 µl TLE (200 mM Tris-HCl pH 8.0, 70 mM EDTA). Additional details and conditions for amplification using the polymerase chain reaction (PCR) are given elsewhere (Godoy and Jordano, 2001).

We used a series of microsatellite primers designed for cultivated *Prunus* species (Abbot, 1998, personal communication; G. King, 1998, personal communication; Cipriani *et al.*, 1999; Downey and Iezzoni, 2000; Sosinski *et al.*, 2000). We tested a total of 43 primers, of which 16 showed polymorphic variation for *P. mahaleb*. We selected a subset of nine primers for use in this study (Godoy and Jordano, 2001).

Statistical analyses

Patterns of fine-scale genetic structure in the tree population were examined using the multilocus microsatellite genotypes of 180 trees based on nine polymorphic loci. A coancestry coefficient, f_{ij}, was estimated between all possible pairs of adult trees genotyped using program f_{ij} *Anal* from J. Nason (Sork *et al.*, 1998). Briefly, f_{ij} measures the correlation in the frequencies of homologous alleles, p_i and p_j, at a locus in pairs of mapped individuals i and j, revealing the degree of genetic similarity for pairs of adult trees growing at different distance intervals. Any spatial pattern of genetic structure in the population will show up in a plot of f_{ij} vs. distance (an autocorrelogram). The coancestry coefficient

is well suited for examining spatial patterns of genetic variation; it assesses the autocorrelation structure of genetic affinity among coexisting individuals (Heywood, 1991; Loiselle *et al.*, 1995a). The significance of f_{ij} values was assessed with randomization tests (Slatkin and Arter, 1991). A plot of f_{ij} values as a function of increasing distance, for both the observed data and the 95% bootstrap estimate derived by randomization ($n = 5000$ resamplings) was examined for significant autocorrelation values at 5 m distance intervals. Assuming no adaptation to the conditions of local microsites, significant f_{ij} values are interpretable in terms of non-random gene flow via pollen and/or seeds, resulting in genetic structuring due to local processes of isolation by distance (Loiselle *et al.*, 1995a).

The relationships between individual dispersed seeds sampled in seed traps and the focal maternal trees were examined by comparing their multilocus microsatellite profiles. For each seed sampled in a seed trap, we examined the match between the multilocus genotype of the endocarp and the multilocus genotype of the focal tree (from leaf tissue) associated with the seed trap. Because the endocarp tissue in *Prunus* is diploid and maternally derived (Roth, 1977), such multilocus genotypes have to be fully matching alleles in all loci.

Matches between the maternal tree and seed-endocarp multilocus genotypes were found and their significance evaluated using the packages Kinship, version 1.3, and Relatedness, version 5.0.5 (Queller and Goodnight, 1989). The method tests the significance of a hypothesized mother–offspring pedigree relationship between a dispersed seed and an adult tree, based on the identity of the endocarp and leaf genotypes, given r_p and r_m, the probabilities that the individuals share an allele by direct descent from their father or mother, respectively. When comparing endocarp and a maternal tree (leaf) tissue, we used $r_p = 1.0$ and $r_m = 1.0$ (K.F. Goodnight, 2000, personal communication; see also Queller and Goodnight, 1989). The test uses the r values, the population allele frequencies and the multilocus genotypes to calculate the likelihood that the genotype combination could have been produced by the relationship specified ($r_p = 1.0$; $r_m = 1.0$). A randomization test is used to assess the significance of the ratio between this likelihood and the one based on a null hypothesis of no relationship. This comparison allowed identification or exclusion of the focal tree as the maternal tree for each seed and the assignment of the maternal tree from the population. The procedure provided significant ($P < 0.001$) and unambiguous assignments for $n = 78$ seeds (82.11% of the seeds sampled).

Results

Patterns of frugivore foraging and the seed shadow

Frugivorous birds feeding on *P. mahaleb* fruits forage non-randomly after leaving fruiting trees, resulting in an extremely patchy pattern of seed rain. Bird preference for patches covered with vegetation, either mid-height shrubs or low shrubs (Jordano and Schupp, 2000), was significant ($\chi^2 = 21.1$, d.f. = 1, $P < 0.0001$); open microhabitats and pines were avoided. In addition, flight distances to the first perch were very short, with 77.5% to perches within 30 m and most species, except *Turdus viscivorus* and *Turdus merula*, perching within 15 m of the focal tree. This resulted in seed densities under shrubs significantly exceeding those in open microhabitats, with the seed rain beneath pines being intermediate. Aggregation of seeds was particularly extreme in the neighbourhood of *P. mahaleb* trees, as a result of both high frequency of departure flights to other *P. mahaleb* trees after feeding and a trend for departure flights to end in perches < 15 m away from any *P. mahaleb* tree (> 92% exit flights of all species except the two *Turdus* species), irrespective of distance (Jordano and Schupp, 2000).

The resulting seed densities differed significantly among microhabitats ($F = 34.65$, d.f. = 8252, $P < 0.0001$), as expected from the highly non-random foraging by the main frugivores. Seed densities in open microhabitats (1.0 ± 0.4, 0.7 ± 0.6, 1.5 ± 0.6 and 5.8 ± 1.1 seeds m^{-2}, for deep soil, gravelly soil, soil with

stones and rocks, respectively; means ± 1 SE) were much lower than those recorded in covered microhabitats (90.9 ± 11.4, 31.5 ± 6.4, 7.7 ± 2.5, 10.5 ± 2.8 and 36.7 ± 6.9 seeds m^{-2}, for *P. mahaleb*, mid-height shrubs, low shrubs, pines with low shrubs and pines, respectively) (Jordano and Schupp, 2000).

Table 20.2 summarizes the results of bird foraging observations and the estimates of specific contributions to the seed rain in different microhabitats. The seed rain to covered microhabitats was typically determined by more than three frugivore species, while seed rain to open microhabitats was determined by one or two species. No single species contributed more than 45% of the seed rain to covered microhabitats, and the seed rain to rocky substrates or beneath pines was determined mainly by *Phoenicurus ochruros* or *T. viscivorus*, respectively (Table 20.2). Because of differences in abundance and visitation rate, some species with low flight frequencies to a particular microhabitat have disproportionately large contributions to the seed rain in those patches. This pattern is illustrated by *T. viscivorus*, with a high contribution to the seed rain beneath *P. mahaleb* or to deep soil patches, despite a low frequency of flights to these microhabitat types. A similar trend occurs for *P. ochruros* to deep soil and rock substrates (Table 20.2).

Seed-addition experiments

The seed-addition treatment had a significant effect on the percentage of plots where at least one seedling emerged ($\chi^2 = 17.9$, $P < 0.0001$): 72.5% of the 'added' subplots recruited at least one seedling, while only 49.3% of the 'control' subplots did so. Among the subplots not recruiting any seedlings (39.1% of the total), 35.2% were seed-addition treatments and 64.8% were controls. The effect of the predator-exclusion treatment was similar; 70.0% of the 'excluded' subplots had at least one seedling emerging vs. 51.9% of the 'control' subplots. The response of seedling emergence to either seed addition or exclusion of post-dispersal seed predators was strongly dependent on the microhabitat type (open or with plant cover) (Fig. 20.2), indicated by a significant microhabitat × addition × exclusion interaction ($F = 8.86$, $P = 0.003$, d.f. = 1312). The effect of seed addition was particularly marked under enclosures beneath shrub cover (Fig. 20.2). When taking into account differences in seedling recruitment among sites, i.e. looking at the proportion of initial seeds that resulted in seedlings, the only significant result was for the exclusion treatment ($F = 26.76$, $P < 0.0001$, d.f. = 1312) and the site × exclusion interaction ($F = 3.83$, $P = 0.048$, d.f. = 1312).

Table 20.2. Exit flight frequencies to different microhabitat types and estimated relative contributions to the seed rain in these microhabitats by avian frugivores visiting *P. mahaleb* trees. For each bird species, figures indicate the percentage of flights to each type of microhabitat and, in parentheses, the estimated percentage of the seed rain in each microhabitat contributed by the species. See Jordano and Schupp (2000) for details.

Species	*Prunus*	Low shrubs	Tall shrubs	Pines*	Deep soil	Rock substrates†
Erithacus rubecula	6.1 (3.7)	43.1 (30.9)	49.3 (19.2)	1.5 (0.2)	0.0 (0.0)	0.0 (0.0)
Phoenicurus ochruros	20.8 (29.4)	4.9 (8.9)	16.8 (16.5)	13.9 (4.2)	1.9 (48.6)	41.7 (86.2)
Sylvia cantillans	28.6 (5.4)	14.3 (3.4)	42.8 (5.6)	14.3 (0.3)	0.0 (0.0)	0.0 (0.0)
Sylvia communis	15.4 (9.8)	34.6 (28.0)	34.6 (15.2)	15.4 (2.2)	0.0 (0.0)	0.0 (0.0)
Sitta europaea	55.6 (4.8)	0.0 (0.0)	11.1 (0.7)	33.3 (0.7)	0.0 (0.0)	0.0 (0.0)
Turdus merula	11.5 (13.5)	19.2 (28.8)	50.0 (40.7)	19.3 (4.6)	0.0 (0.0)	0.0 (0.0)
Turdus viscivorus	6.5 (33.4)	0.0 (0.0)	0.6 (2.1)	85.8 (87.8)	0.6 (51.4)	6.5 (13.8)

*Includes pines with and without low shrubs beneath.
†Includes gravelly soil, rocks with soil and rock substrates.

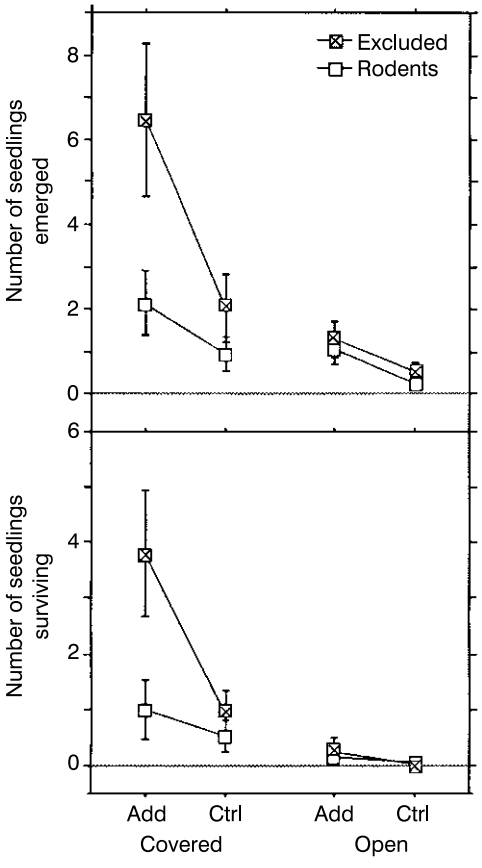

Fig. 20.2. Interaction charts for seed-addition experiments with *P. mahaleb* seeds sown in 'covered' microhabitats (combining sites beneath *P. mahaleb* trees and sites beneath pine trees with low juniper shrubs) and 'open' microhabitats (including sites with grassy cover on deep soil; and sites with gravelly and rocky soil). Shown are the mean number of seedlings emerged (± 1 SE) and mean number of seedlings surviving after the first summer (± 1 SE) in experimental blocks that combined the following treatments: 'control' seed density ('Ctrl') – the median seed density sampled with seed traps – and 'added' seed density ('Add') – the 95 percentile of the median density for each microhabitat type, i.e. approximately a threefold increase in the number of seeds sown. Each block included these treatments within exclusions of post-dispersal seed predators (rodents) ('Excluded') and in plots accessible to them ('Rodents').

DNA extraction and amplification from individual seeds

To check the accuracy of leaf and endocarp tissue comparison, endocarps and embryos of progeny obtained in diallel crosses (P. Jordano, unpublished data) were genotyped and compared to the genotype of their corresponding sire and dam trees. The multilocus genotypes of the seed endocarps were identical to those from leaves of the mother tree, as expected from the anatomical origin of the endocarp tissue, which, in *Prunus* drupes, derives from the carpelar wall (Roth, 1977) and is therefore diploid and maternally derived (Table 20.3; Godoy and Jordano, 2001). Therefore, the endocarp genotype of any *P. mahaleb* dispersed seed can be used to unambiguously identify its source tree. On the other hand, the genotypes of embryos were compatible with those of their sire and dam trees, i.e. for every locus one allele was contributed by the mother and the other by the father. Interestingly, by comparing the genotypes of the embryo and the endocarp of a dispersed seed, the haplotype of the male gamete can be easily and unambiguously inferred and the males with compatible genotypes in the population can be identified as putative fathers. Additional genotyping of the embryo can thus be used to identify the siring tree of dispersed trees with higher exclusion probabilities than possible if the mother were not identified. This approach would allow the concurrent analysis of seed dispersal and pollination and thus seed- and pollen-mediated gene flow (J.A. Godoy and P. Jordano, in preparation).

We were able to unambiguously assign the maternal trees for $n = 78$ seeds (82.1%); the maternal trees for the remaining 17 seeds may have been an unsampled adult tree from the local population or a tree from another population. As far as we know, our sampling of the adult trees in Nava de las Correhuelas was complete. Thus, we attribute this fraction of unassigned seeds (17.9%) to immigrants from other populations.

Local genetic structuring of adult trees

Significant genetic structure was evident in the spatial autocorrelation of the derived estimate of the coancestry coefficient among pairs of adult trees (Fig. 20.3). In particular, there was a significant peak in autocorrelation at the interval of 0–20 m. These results parallel those obtained previously with RAPD markers in the same population (Jordano, 2001); *Prunus* trees with close genetic distance grow close together. The autocorrelation coefficient between the genetic distance matrix derived from RAPD markers and a 'hypothesis' matrix

Table 20.3. Example of polymorphic variation in nine simple sequence repeat (SSR) or microsatellite loci of *P. mahaleb* leaves (maternal tree number 1927) and both endocarp and embryo tissues of two of its seeds. The size of alleles for each of the nine SSR loci is given for the leaf, endocarp and embryo tissue. Alleles are designed by the size (bp) of their products. In all cases the multilocus genotype for the endocarps matches the leaf genotype, as expected from the diploid maternal derivation of the endocarp tissue; however, the genotypes of the two embryos differ from either the maternal tissues of leaves and endocarps, with unmatching alleles shown in bold type. See Godoy and Jordano (2001) for details.

Locus	Leaves	Endocarp no. 1	Endocarp no. 2	Embryo no. 1	Embryo no. 2
UDP96-001	124/124	124/124	124/124	124/124	124/124
pchgms3	179/191	179/191	179/191	**191**/191	179/191
UDP96-018	246/246	246/246	246/246	246/246	246/246
UDP97-403	107/107	107/107	107/107	107/107	107/107
PS12A02	175/185	175/185	175/185	**185**/185	**175**/175
pchcms5	233/235	233/235	233/235	**235**/235	**235**/235
UDP98-406	98/102	98/102	98/102	**102**/102	98/102
UDP97-402	148/152	148/152	148/152	**148**/148	148/152
MS01A05	200/200	200/200	200/200	200/200	200/200

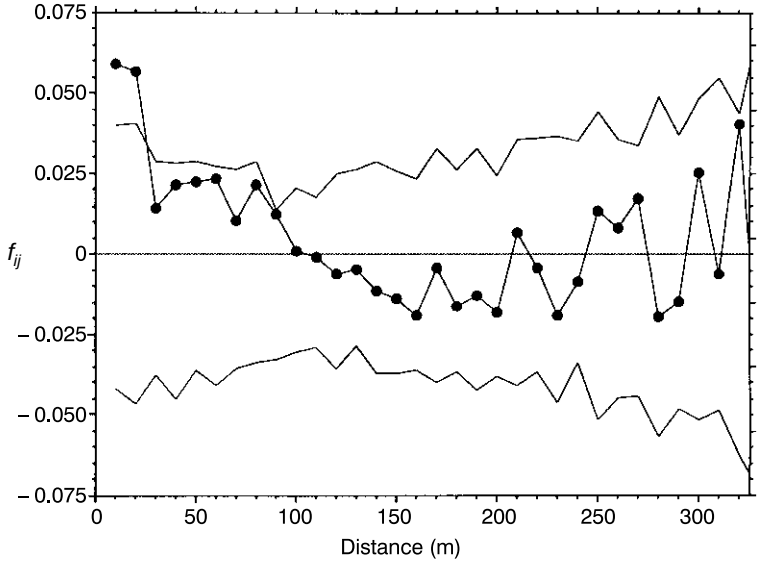

Fig. 20.3. Spatial autocorrelogram for the estimated coancestry values (f_{ij}) between pairs of adult *P. mahaleb* trees (n = 180) within 5 m distance intervals in the Nava de las Correhuelas population. Thin lines represent the bootstrap 95% confidence intervals about the hypothesis of no spatial genetic structure. High f_{ij} values outside the confidence intervals indicate significant positive autocorrelation in genetic similarity among individuals located up to 20 m apart; coancestry values were not significantly different from zero beyond this distance class.

about neighbourhood structure (i.e. trees located in the same patch having distance equal to zero, those growing in different patches having distance equal to one (see Jordano, 2001)) was significant ($r = 0.467$, $P = 0.044$, $n = 10{,}000$ randomizations; Mantel's test (Casgrain and Legendre, 1998)). Both microsatellite and RAPD data show a significant effect of isolation by distance in a pattern that parallels the strong structuring of the seed shadow generated by frugivores.

Maternity assignments for progeny of 'focal' trees

Focal trees differed in the proportion of their own seeds beneath their canopies (Fig. 20.4). For some trees most seeds deposited by frugivores beneath the canopy were their own. This contrasted with other trees in which seeds voided by frugivores were from several maternal trees. On average, 2.8 (range, 1–4) maternal trees contributed seeds beneath a given *P. mahaleb* tree (Table 20.4), a type of 'autodispersal' in which seeds are delivered away from the mother tree but beneath the canopy of conspecifics. When estimated over the entire sample, 70% of assigned seeds were located beneath their maternal plants (Table 20.4). Forty per cent of assigned seeds sampled beneath mid-height shrubs were from nearby focal trees. The proportion of seeds beneath mid-height shrubs and pine trees that were assignable to focal trees decreased dramatically at longer distances (> 10 m away from the focal trees) (Table 20.4). Sampling locations beneath mid-height shrubs had similar numbers of distinct maternal trees contributing seeds, whereas locations under pines had fewer contributing maternal trees at greater distances (Table 20.4). A relatively high number of maternal trees (up to five trees) contributed seeds to areas beneath shrub cover, irrespective of distance to source trees. The number of trees contributing seeds to a particular sampling location was positively correlated with the number of reproductive trees within 15 m radius of the sampling location ($r_s = 0.526$, $P = 0.002$, $n = 19$), an expected result given the markedly restricted flight patterns of birds after feeding in fruiting *P. mahaleb* trees.

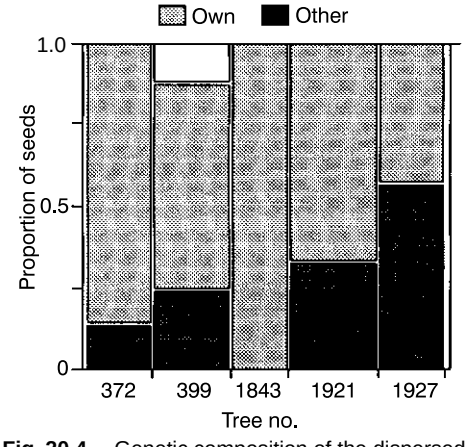

Fig. 20.4. Genetic composition of the dispersed seed shadow in seed traps beneath five 'focal' *P. mahaleb* trees. Seeds are divided into 'own' progeny (light grey) – seeds from the maternal focal tree – and those from 'other' trees (dark grey), mostly in the neighbourhood of the focal tree. The blank area represents unassigned seeds. Seeds were assigned to a particular maternal tree on the basis of the relatedness value estimated from the multilocus microsatellite genotype of the seed endocarp compared with the multilocus microsatellite genotypes of 180 *P. mahaleb* reproductive trees in the population.

Discussion

Seed dispersal by animals is a key process in plant population dynamics, one that subsumes both demographic and genetic effects. Our results show that frugivore activity can severely limit plant population recruitment and strongly influence local genetic structure. The main mechanism driving this process is frugivore foraging behaviour. In particular, the non-random pattern of frugivore movement in heterogeneous landscapes imposes markedly non-random patterns of seed rain among distinct microhabitat types. This, together with the fact that a given frugivore disperses many seed species, makes the seed rain generated by all frugivores highly heterogeneous and aggregated. Thus, most sites in the landscape receive few or no seeds despite copious fruit production and thorough fruit

Table 20.4. Summary of the genetic composition of the seeds sampled in seed traps at different replicate sampling locations for five types of microhabitats. Sampling sites away from *P. mahaleb* were located in relation to each of five 'focal' trees at close (< 10 m) or far (> 10 m) distance. Sites with *Pinus* were all > 10 m away from the focal tree.

Microhabitat type	No. of replicate locations (no. of seeds)*	'Own' progeny†	No. of trees contributing progeny‡	Distance to nearest *Prunus*§	No. of *Prunus* < 15 m‖
Beneath *Prunus*	5 (37)	26 (0.7)	2.8 (1–4)	0 m	2.8 (1–5)
Mid-shrubs, < 10 m	5 (31)	12 (0.4)	2.8 (1–5)	< 10 m	3.8 (3–5)
Mid-shrubs, > 10 m	4 (19)	1 (0.05)	3.0 (1–3)	> 10 m	6.8 (4–9)
Pinus + low shrubs	2 (4)	0 (0.0)	2.0 (1–2)	> 10 m	1.0 (1)
Pinus	3 (4)	0 (0.0)	0.7 (0–1)	> 10 m	0.3 (0–1)

* Number of replicate sampling locations of each microhabitat type and number of seeds genotyped. The number of replicate locations and type of microhabitat sampled for each tree varied depending on the characteristics of the site.
† Number (and proportion) of seeds assigned to the maternal focal tree.
‡ Mean number (range) of distinct maternal trees contributing progeny to the sampling location.
§ Distance category to focal tree.
‖ Mean number (range) of reproductive *P. mahaleb* trees within 15 m radius of seed-sampling location.

removal, a phenomenon that Jordano and Schupp (in prep.) define as dissemination limitation (Table 20.1).

Dissemination limitation is a rather general characteristic of frugivore-generated seed shadows (Wenny and Levey, 1998; Wenny, 2000; see also Schupp *et al.*, this volume). Most studies on this topic have focused on distance-restricted dispersal (e.g. Clark *et al.*, 1999a); consequences for plant population dynamics deserve further study. First, the fact that the diversity of frugivore species contributing seeds to a particular patch in the forest can vary depending on the type of microhabitat has potential consequences for population structure. Secondly, marked peaks and valleys in the landscape pattern of seed distribution, which depend on the particular landscape of the site, indicate that recruitment can be limited by seed dispersal. Potentially, seeds cannot reach microhabitats where establishment probability will be high. Thirdly, animal-created seed shadows can result in marked local genetic structuring of the population, which can then influence gene flow and recruitment.

Most recent analyses of endozoochorous seed dispersal focus on seed rain resulting from all dispersers. Disclosing the unique contributions of each disperser species to a seed shadow can only be completed by combining simultaneous analysis of frugivore activity, post-foraging movements and seed rain patterns (e.g. Wenny and Levey, 1998; Jordano and Schupp, 2000). When this is accomplished, it becomes evident that the seeds contributed to different portions of the seed shadow are delivered by different frugivore species. As a result, population recruitment can be attributable to the activity of only a limited set of species within a diverse frugivore assemblage. Thus these recent studies with Mediterranean and neotropical frugivorous birds have shown that, despite a high diversity of interactions, plant–frugivore mutualisms can be directed by a few key interactions, with disproportionate effects on seed recruitment.

Uneven seed delivery patterns invariably result in extremely heterogeneous seedling recruitment patterns. For both *P. mahaleb* and *Ph. latifolia* (Jordano and Herrara, 1995), most locations in the landscape had very small ratios of seedlings recruited to seeds dispersed. The main factor influencing this limited recruitment depended on microhabitat type. In general, dissemination limitation was higher in open microhabitats, where lack of seed arrival resulted in patches where seedling recruitment was impossible. Post-dispersal factors influencing germination and/or seedling survival can be more limiting in covered microhabitats, where seed delivery by frugivores is frequently high. For example, covered microhabitats

for *P. mahaleb* typically show very high seed predation rates but high seedling survival rates (Schupp, 1995; Hulme, 1997). Whenever frugivorous birds are the primary dispersers, we might expect seed delivery to covered microhabitats and avoidance of more open patches, resulting in aggregated seed rain. In covered microhabitats, our seed-addition treatments disproportionately increased both seedling emergence and seedling survival only when seed predators were excluded, suggesting that post-dispersal seed predation, not failure of seeds to arrive, limits recruitment in these microhabitats. In open microhabitats, the addition of seeds resulted in more seedlings initially but not after the first summer drought. This suggests the final establishment of seedlings is strongly limited by adverse abiotic conditions, not by dissemination limitation. The significant three-way interaction of microhabitat type, seed-addition treatment and predator exclusion indicates that the influence of seed dispersal on initial seedling establishment in this system is strongly dependent on microhabitat type. Our central conclusion is that seed-dispersal limitation cannot be seen as a population-wide process unless one considers the summed contributions to recruitment of all patches.

Our genetic analyses demonstrate that frugivores also dramatically influence the spatial pattern of dispersed genotypes. Frugivores generate two types of shadows: seed shadows and genotype shadows. Our analysis of microsatellite loci variation reveals that genotype shadows can be as aggregated as seed shadows. First, the adult population showed a dramatic spatial pattern of genetic structure, with a strong peak in genetic similarity near (< 20 m) conspecifics, consistent with the pattern of seed rain estimated from observations of frugivorous birds (Jordano and Schupp, 2000). This short distance peak was also evident from RAPD analyses (Jordano, 2001) and confirms previous results with other animal-dispersed species (Loiselle *et al.*, 1995a; Aldrich *et al.*, 1998; Schnabel *et al.*, 1998b; Ueno *et al.*, 2000). Strongly distance-limited seed shadows, coupled with spatially aggregated seed delivery, probably result in highly structured genetic diversity within populations of animal-dispersed species. It is interesting that animal-dispersed species exhibit a very high proportion of genetic variation within populations, typically > 70%, as determined by allozyme data or analysis of molecular variance (AMOVA) (Hamrick *et al.*, 1993; Nybom and Bartish, 2000). Thus, despite the fact that individual populations of animal-dispersed species 'capture' most of the genetic diversity found at the regional level, our results show that patches of neighbouring adult trees can be very genetically homogeneous. This pattern is probably attributable to the effects of isolation by distance operating at the within-population level and, in the case of *P. mahaleb*, is associated with the short-distance movements made by frugivores near fruiting trees (Jordano and Schupp, 2000).

A technical advance of our research is the ability to link genotypes of the maternal trees with those of dispersed seeds (also see Godoy and Jordano, 2001). By focusing on 'focal' trees we corroborated a strongly contagious pattern of seed delivery; most seeds of a given plant can be found either beneath its canopy or in the immediate vicinity (in our system, under covered microhabitats within 10–15 m). Seeds from our focal trees did not appear in seed traps located > 15 m away or in microhabitats not frequented by frugivores. Previous analyses of seed shadows have likewise shown very limited dispersal distances and contagious seed delivery (Murray, 1988; Mack, 1995; Levey and Sargent, 2000) but may fail to detect long-distance dispersal events, which can now be tracked with genetic markers. Our analyses of genotypes of dispersed seeds in this population revealed disproportionately frequent short-distance dispersal events (< 5 m), combined with extremely infrequent events of long-distance dispersal (> 250 m) (Godoy and Jordano, 2001). We were able to assign maternity for 82.1% of the seeds sampled and estimate that most of the remaining 17.9% are attributable to long-distance dispersal from other *P. mahaleb* populations in the region.

We found enormous variation among focal trees in the genetic composition of the seeds delivered by frugivores beneath their canopies. Individual fruiting trees not only receive dispersed seeds from their own canopy, but also receive seeds dispersed from conspecific trees. We consider this to be a sort of

'autodispersal'; our data reveal that some trees can act as 'sinks' for seeds from other trees. For example, we found up to 60% of seeds from other trees beneath the canopy of particularly attractive trees, while other fruiting *P. mahaleb* had no seeds from neighbours or, typically, < 20%. These results, combined with our finding that up to five distinct maternal trees can contribute seeds to a single patch, suggest a complex pattern of overlapping seed shadows from different individual trees. Clearly, theoretical models attempting to estimate seed shadows by extrapolating from linear dispersal distances should take into account this intrinsic complexity of animal-generated seed shadows (Clark *et al.*, 1999a,b). This could be accomplished with relative ease for each sampling location by including estimates of the minimum number of maternal trees expected to contribute progeny, given the distribution of distances to adult trees and the type of microhabitat in the sampling location. Our preliminary data (Table 20.4; see also Godoy and Jordano, 2001) indicate that up to five or six trees can contribute progeny beneath covered microhabitats close to a fruiting tree, and up to three trees can contribute seeds to similar habitats at more distant locations from these microhabitats; finally, up to two trees can contribute seeds to less preferred microhabitats.

The use of molecular techniques to assess maternity of dispersed seeds offers new avenues for research in plant–frugivore mutualisms. Direct assessment of dispersal distances, analysis of the genetic diversity and make-up of the seed shadow at a microscale and estimation of both demographic and genetic effects of frugivores that differ in foraging modes are possible by using hypervariable markers, such as microsatellites. We see the most promising approach as the one that combines such data with careful observations of frugivore behaviour and demographic analyses of the stages in plant recruitment.

Conclusion and Perspectives

The activity of frugivores simultaneously influences the number and genetic make-up of seeds dispersed across the landscape. These two components of the seed shadow set the template for plant recruitment. Approaches to plant–frugivore interactions have moved towards integrating the net effects of frugivores on the entire sequence of recruitment stages following seed delivery (Schupp, 1993). Recent developments in molecular hypervariable markers allow unambiguous assessment of paternity and kinship relationships for seeds obtained in studies of seed rain, providing a powerful tool for directly assessing dispersal distances and spatial patterns of dispersal (Ouborg *et al.*, 1999; Godoy and Jordano, 2001). The key issue of whether frugivore activity limits dispersal in plant populations and constrains plant population dynamics can now be rigorously assessed. The natural recruitment cycle of many plant species can be seed limited, especially in situations of dramatic disturbance, such as severe habitat fragmentation. Local extinction of isolated populations in fragments can result not only from demographic bottlenecks originating from failure to disperse seeds, but also from severe genetic bottlenecks. If strongly aggregated seed dispersal of endozoochorous species is typical, then fragmentation will result in spatial isolation of close relatives, leading to severe reduction of genetic diversity. 'Traditional' approaches to the study of plant–frugivore interactions (Snow and Snow, 1988) are essentially blind to such scenarios and should be combined with both demographic and genetic studies to fully understand how frugivores affect plant communities.

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