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RAPD variation and population genetic structure in *Prunus mahaleb* (Rosaceae), an animal-dispersed tree

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Abstract

We examined the patterns of random amplified polymorphic DNA (RAPD) variation among seven *Prunus mahaleb* (Rosaceae) populations extending over ≈ 100 km² to examine local differentiation in relation to spatial isolation due to both geographical distance and differences in elevation. No less than 51.4% of the RAPD loci were polymorphic, but very few were fixed and among-population variation accounted for 16.46% of variation in RAPD patterns. Mean gene diversity was 0.1441, with mean Nei's genetic diversity for individual populations ranging between 0.089 and 0.149. Mean G_{ST} value across loci was 0.1935 (range, 0.0162–0.4685), giving an average estimate for *Nm* of 1.191. These results suggest extensive gene flow among populations, but higher G_{ST} and lower *Nm* values relative to other outcrossing, woody species with endozoochorous dispersal, also suggest a process of isolation by distance. The combined effect of both geographical and elevation distances and nonoverlapping flowering and fruiting phenophases on the G_{ST} matrix was partially significant, revealing only marginal isolation of the *P. mahaleb* populations. The matrix correlation between estimated *Nm* values among populations and the geographical + elevation distance matrices ($r = -0.4623$, $P = 0.07$), suggests a marginal trend for more isolated populations to exchange less immigrants. Long-distance seed dispersal by efficient medium-sized frugivorous birds and mammals is most likely associated to the high levels of within-population genetic diversity. However, vicariance factors and demographic bottlenecks (high postdispersal seed and seedling mortality) explain comparatively high levels of local differentiation.

Keywords: avian frugivores, genetic diversity, plant recruitment, RAPD, seed dispersal, spatial variation

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Introduction

Frugivorous animals that disperse the seeds of fleshy-fruited plant species can potentially exert a dramatic influence on the spacing patterns, neighbourhood density of conspecifics, and overall population structure of the plants. As a result of their foraging activity, the initial template for plant regeneration is defined as a seed shadow that represents the starting point for population recruitment across the landscape (Jordano 1992; Schupp 1993). Factors acting after seeds are delivered, such as differential mortality caused by postdispersal seed predators, differential germination and early seedling survival, can either erase or maintain this initial landscape pattern of recruitment. The net result depends on contrasting selective effects which are specific to both the recruitment stage and the particular microhabitat patch where the seeds were delivered (Jordano & Herrera 1995; Schupp & Fuentes 1995; Schupp 1995). Considerable effort has been dedicated to sorting out the net demographic consequences of frugivore activity on plant demography (Howe *et al*. 1985; Schupp 1993; Herrera *et al*. 1994), yet the potential consequences for the genetic make-up and structuring of the populations and potential influences on gene flow via seed movement have been largely unexplored despite recent interest and pioneer work (Hamrick *et al*. 1993; Gibson & Wheelwright 1995; Loiselle *et al*. 1995a; Nason

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et al. 1996; Epperson & Alvarez-Buylla 1997; Nason *et al*. 1998; Schnabel *et al*. 1998). When populations are structured in space (e.g. fragmented populations, metapopulations), animal frugivores can have an impact on among-population gene flow via seed (Nason & Hamrick 1997; Sork *et al*. 1999). The information on frugivore movements at this scale is extremely insufficient due to intrinsic limitations of direct monitoring of animal movement at a regional scale, yet there is evidence for the direct effect of frugivore movement on among-population dispersal of seeds (Loiselle *et al*. 1995a,b; Chase *et al*. 1996; Fragoso 1997; Julliot 1997; Nason & Hamrick 1997).

Recent analyses of among-population patterns of genetic variation have emphasized the correlates of the partition of among-population genetic variance with specifics of the breeding system, especially outcrossing rates (Hamrick & Godt 1997), and pollination-mediated gene-flow (Sork *et al*. 1998). However, seed dispersal patterns can also have a dramatic influence in the partitioning of genetic variation within and among populations, as demonstrated by intensive research with allozyme markers (Hamrick *et al*. 1993; Hamrick & Godt 1997). These studies revealed zoochorous plant species to have characteristically high levels of within-population genetic variation in comparison with other seed-dispersal syndromes. Nevertheless, no comparative analysis to date has pointed out peculiarities of pattern variation at the molecular level for animal-dispersed species. Typically, animal-dispersed species show moderate to large within-population component of genetic variation, associated with extensive gene flow via seed dispersal in addition to outbreeding via pollen flow (Hamrick *et al*. 1993). The well established nonrandom association of woody habit, predominately outcrossed insect pollination, and endozoochorous seed dispersal (Eriksson & Bremer 1992) would result in a combination of low G_{ST} values and high frequency of immigrant exchange among populations. However, the among-population variation component can be significant despite being reduced (Bussell 1999), and we know almost nothing about its ecological correlates. For example, despite extensive foraging areas of frugivorous animals, especially the larger-sized species, seed dispersal between populations can be very low if the fruiting phenologies are nonoverlapping (e.g. due to altitudinal differences) and the fruit is an important component of diet (Wheelwright 1983). If frugivores are tracking the availability of ripe fruits, they would restrict seed movement to those patches or populations where ripe fruits are available concurrently, especially if no other species is available (see e.g. Herrera & Jordano 1981 for *Prunus mahaleb*). Two populations or fragments located at different elevation would show nonoverlapping flowering and fruiting periods, thus hindering the possibilities for gene flow between them. Thus, geographical distance is not the only factor influencing isolation by distance, as the complex spatial structuring of a mosaic of subpopulations would also have an important influence (Sork *et al*. 1999). In addition, factors involved in vicariance events could have an important effect in populations of long-lived tree and shrub species with the characteristic combinations of life-history and reproductive traits mentioned above, determining nonuniform genetic make-up throughout a species range (Bossart & Prowell 1998).

In this paper we analyse the patterns of random amplified polymorphic DNA (RAPD) variation in an animal-dispersed, fleshy-fruit producing rosaceous tree, *Prunus mahaleb*, for which detailed information on long-term patterns of variation in fruit traits and ecological patterns of interaction with pollinators, seed dispersers, and herbivores are available (Jordano 1993, 1994, 1995; Jordano & Schupp 2000). The species shows a highly fragmented distribution in the south-east Spanish mountains as a result of the postglacial shifts in distribution and its relict status (Hegi 1995), now subject to specific protection and management plans. Previous genetic analyses with *Prunus* species involved chiefly the identification of cultivar affinities (e.g. Heinkel *et al*. 1998), quantification of genetic diversity in rootstocks (Warburton & Bliss 1996; Casas *et al*. 1999), and linkage mapping of fruit-related traits (Wang *et al*. 1998), but few data are available on the natural levels of genetic variation in wild populations (Dawson & Powell 1999) and their ecological correlates.

RAPD markers (Fritsch & Rieseberg 1996) have been extremely useful to assess patterns of genetic diversity in a number of plant species (Bussell 1999; Ouborg *et al*. 1999). Despite important limitations due to their dominant nature, RAPD markers can be used to assess fixation indexes and population genetic parameters when appropriate statistical analysis is used (Huff *et al*. 1993; Lynch & Milligan 1994; Stewart & Excoffier 1996). However, there are very few data available on the patterns of RAPD variation for predominantly outcrossed, animal-dispersed, woody species in the wild; only five species with endozoochorous seed dispersal were included in the 37 species studied for RAPD variation reviewed by Bussell (1999).

Our main objective was to complete a dissection of the genetic components of variability among and within populations of *P. mahaleb* trees by focusing on a limited set of fragmented populations where other characteristics of the reproductive cycle of the plant and its interaction with frugivores are well documented. If isolation by distance is an ongoing process in this set of populations, we should be able to assess the relative role of geographical distance, isolating barriers such as watersheds, and differences in phenology (correlated with isolation in elevation) in determining the patterns of genetic similarity among populations.

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-east Spain, 37°59′ N, 2°54′ W), with most data obtained during 1997–99. Seven different populations were sampled in the following locations (with elevation and UTM geographical coordinates): Roblehondo (1300 m, WG1301), Torcal del Cerecino (1480 m, WG1395), Cañada de la Medianega (1540 m, WG0794), Nava Noguera (1550 m, WG1799), Calarilla (1590 m, WH1300), Nava de las Correhuelas (1615 m, WG1298) and Cabeza del Tejo (1635 m, WG1095). This relatively narrow elevation gradient has an associated variation of 5–9 °C in mean weekly minimum temperature and 4 °C in mean maximum temperature during the reproductive period of *Prunus mahaleb*, marking a sharp variation in the flowering and fruiting phenophases (P. Jordano, personal observation). All the sites share the general physiognomy and vegetation composition characteristic of relatively well-preserved south-eastern Spanish Mediterranean mountain vegetation on calcareous substrate (Valle *et al*. 1989), and detailed descriptions have been published elsewhere (Herrera 1989; Jordano 1995; Jordano & Schupp 2000). Two main vegetation types are usually present at the sites. Deciduous vegetation dominates deep soils with deciduous treelets and shrubs of temperate affinity, while adjacent rocky exposed slopes are dominated by open pine forest (*Pinus nigra*, ssp. *salzmannii*) with juniper (Valle *et al*. 1989). *P. mahaleb* occurs scattered as small isolated patches or populations of < 20 individuals and a few large populations with > 200 trees within a continuous matrix of mixed open pine forest.

P. mahaleb is a small tree (2–10 m height) growing scattered at mid-elevations (1200–2000 m) in south-eastern Spanish mountains, extending through central and eastern Europe to west-central Asia (Webb 1968). The species is included within subgenus *Cerasus*, with typically diploid ($2n = 16$) taxa like the sweet cherry (*P. avium*), which is supported by recent phylogenetic work on cpDNA (Badenes & Parfitt 1995). Subgenus *Cerasus* is the most divergent and constitutes a monophyletic group diverging early in *Prunus* radiation.

P. mahaleb has insect-pollinated flowers, and approximately equal proportions of solitary bees and flies act as pollinators (see Jordano 1993 for details). Flowers are hermaphrodite, although male sterility has been reported for these populations (Jordano 1993); the frequency of male-sterile trees ranges between 8% and 50% in the studied populations. Although outcrossing rates estimated from seed mass data obtained in experimental pollinations were high ($S \approx 0.53$), and certainly male-sterile trees are obligately outcrossed, selfing is frequent in hermaphrodite trees and results in lower fruit-set and small fruit and seed size (Jordano 1993). Trees fruit every year but with marked variation in fruit crop size both within and among populations (P. Jordano, personal observation). The local populations studied grow as discrete patches of trees scattered over variable extensions, usually 0.2–2 km² in the southernmost part of the park. They are representative of other populations in south-eastern Spanish mountains (P. Jordano, personal observation), usually with frequent small populations and scarce large ones.

Birds and mammals are frequent consumers of *P. mahaleb* fruits during mid-summer and disperse its seeds. At least 28 bird species, four mammals, and one lizard have been recorded feeding on the fruits at the study sites (P. Jordano & E.W. Schupp, personal observation). Frugivorous birds visiting *P. mahaleb* trees in Spanish populations usually behave as legitimate seed dispersers (warblers, *Sylvia* spp.; robin, *Erithacus rubecula*; thrushes, *Turdus* spp.; and redstarts, *Phoenicurus* spp.), swallowing the fruits whole and defecating and/or regurgitating the seeds, usually after leaving the tree. Some species behave as pulp consumers (tits, *Parus* spp. and chaffinch, *Fringilla coelebs*) pecking the fruit, tearing-off the pulp and dropping the seed to the ground beneath the parent tree (Jordano 1994, 1995; Jordano & Schupp 2000). Large-bodied species (> 100 g) such as thrushes, woodpeckers (*Dendrocopos major*), corvids (*Corvus* spp.), and pigeon (*Columba palumbus*) can act as long-distance seed dispersers in the area, as long departure flights away from the feeding trees have been recorded (Jordano & Schupp 2000). Carnivorous mammals, including red foxes, stone martens, and badgers, usually take *P. mahaleb* fruits from the ground and disperse large numbers of seeds/scat (J.L. García-Castaño & P. Jordano, personal observation). They can also disperse *P. mahaleb* seeds to long distances due to long retention times of seeds in the gut (Herrera 1989).

Most seed rain of *P. mahaleb* in the study areas is contributed by frugivorous birds (J.L. García-Castaño & P. Jordano, personal observation). Seed rain and the resulting recruitment pattern of seedlings and saplings are highly patchy, and largely restricted to covered microhabitats beneath woody cover in the vicinity of fruiting trees (Jordano & Schupp 2000).

Phenological observations

To assess the degree of phenological similarity in flowering and fruiting periods among *P. mahaleb* populations one of us (PJ) kept records of the phenological stage of individually marked trees in weekly visits during the reproductive period, monitoring all the trees when < 20 individuals were present or at least 35 trees in the larger populations. A population-wide score of phenological stage was

Locality	Elevation (m)	Number of trees sampled	Estimated fraction of adult population sampled
Low elevation			
Roblehondo (A)	1300	11*	73.3
Torcal del Cerecino (B)	1480	26	< 10.0
Mid-elevation			
Cañada Medianega (C)	1540	15	78.9
Nava Noguera (D)	1550	20	< 5.0
Calarilla (E)	1590	25	78.1
Nava Correhuelas (F)	1615	32	35.0
High-elevation			
Cabeza del Tejo (G)	1635	15	88.2

*Six adult trees and five saplings.

Table 1 Sampling localities and sample size of *Prunus mahaleb* material used in this study. All localities are within Parque Natural de las Sierras de Cazorla, Segura y Las Villas, Jaén province, south-east Spain. Letters refer to Fig. 1

assigned from the individual tree scores (see Stiles 1975). The dates when first and last flowers or fruits were available, in peak bloom or ripening, or when > 50% of individuals were in peak phenophase were determined. These scores were used to compute the degree of phenological overlap between pairs of populations as simply the proportion of dates when both were with > 50% of individuals in peak phenophase.

Field sampling

Fresh leaf tissue was sampled from a total of 144 trees in the seven study populations during mid-May 1998. Material was sampled from adult reproductive trees in all populations except Roblehondo, where five saplings were sampled in addition to trees. Individual trees were selected for sampling from those previously marked in a long-term study of fruit traits and ecological interactions with frugivores (Jordano 1995; Jordano & Schupp 2000; P. Jordano in prep.). Sampled trees were spaced within 300–500 m in each population, usually within 150 m in the smaller populations. In the small populations, almost all the trees present were sampled (up to 88% of the adult trees); in the larger populations (N. Noguera), trees were haphazardly selected from within a restricted area where marked trees were growing (Table 1). In the N. Correhuelas population, 32 trees were selected at random from a larger set of 72 marked trees.

For each tree, discs were punched from 5 to 10 young expanding leaves (0.8–1.5 cm long) chosen haphazardly at different positions of the canopy, kept within labelled, duplicate, Eppendorf tubes, quick frozen in liquid nitrogen and stored at –80 °C.

DNA extraction and amplification

DNA was extracted from 100 to 200 mg of fresh leaf tissue using the rapid miniprep method of Cheung *et al*. (1993). Briefly, tissue was homogenized in 320 µL of extraction buffer (200 mM Tris-HCl pH 8.0, 70 mM EDTA, 2 M NaCl, 20 mM sodium bisulfite) 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. 200 µL of the supernatant was transferred to a new tube and 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 pellet was washed with 70% ethanol, dried and resuspended in 100 µL TE buffer. In our hands this extraction procedure yielded 10–15 µg DNA and we have checked its stability by successful amplification and repeatable RAPD banding patterns after two years storage at –20 °C.

The concentrations of $MgCl_2$, dNTPs, primer, and template DNA in the reaction were established according to an orthogonal optimization protocol, as suggested by Cobb (1997). DNA extracts were diluted 1:20 in water and 5 µL of this dilution was used as template in a polymerase chain reaction (PCR) containing 67 mM Tris-HCl pH 8.8, 16 mM $(NH_4)_2SO_4$, 3.2 mM $MgCl_2$, 0.01% Tween-20, 0.01% BSA, 0.15 mM of each dNTP, 0.375 µM of the arbitrary decamer, and 0.75 U of *Taq* DNA polymerase in a total volume of 10 µL. PCR reactions were carried out in a MJ Research PTC-100 thermocycler programmed for an initial denaturation step of 94 °C for 1 min, followed by 45 cycles of 10 s at 94 °C, 10 s at 36 °C and 70 s at 72 °C. The reaction was completed with a final run at 72 °C for 2 min. Amplification products were separated on 1.4% agarose gels in 1× TBE buffer. Ethidium bromide was included in both the gel and the electrophoresis buffer at a concentration of 0.3 µg/µL. Gels were run for 3 h at 50 mA and visualized under UV light. Gel images were digitized with a high-resolution digital camera for later scoring and analysis.

Data analyses

RAPD banding patterns. We initially tested 40 primers (Opéron, sets C and D) on two individuals for each of the seven populations. We selected 12 primers with both reproducible and polymorphic variation with well defined and darkly staining bands. Band scoring was performed simultaneously and cross checked by both PJ and JAG on a total of 142 markers. Of them, 73 were polymorphic, accurately scorable, showed high readability, and were repeatedly visible. Repeatability of the banding patterns was checked for each primer on several samples with independent DNA extractions, and a repeatability test sample was included in each amplification reaction. Only those RAPD markers that reproduced consistently across successful PCR reactions and across DNA extractions were included in analysis.

Amplification products were scored as discrete, binary states (present/absent) for each individual tree and labelled by primer and estimated band size. As medium to high outcrossing rates are expected in *P. mahaleb* progeny (Jordano 1993), genetic diversity statistics applied to RAPD data would underestimate diversity due to the dominant character of RAPD markers yielding lower estimates when compared to estimates derived from codominant markers such as isozymes (Fritsch & Rieseberg 1996). Thus, we included a large sample of trees in our analyses and avoided using bands with frequency higher than $1 - (3/n)$, where n is the number of individuals in analysis (Lynch & Milligan 1994).

Statistical analysis. RAPD markers were used to assess genetic diversity patterns in the *P. mahaleb* populations. RAPDs are generally considered nuclear and show Mendelian segregation (Heun & Helentjaris 1993), but their dominant nature precludes the estimation of population genetic statistics such as allele frequencies and fixation values. However, modifications proposed by Stewart & Excoffier (1996; see also Excoffier *et al*. 1992) based on RAPD profile similarity allow the estimation of these basic parameters (see, e.g. Palacios & González-Candelas 1997). Estimates of fixation indexes based on dominant markers like RAPDs rely on the unrealistic assumption of Hardy–Weinberg equilibrium ($F_{IS} = 0$) in the populations examined. However, using empirically derived values of F_{IS}, it is possible to obtain robust genetic parameters with RAPD markers. We used observed estimates of outcrossing rates for *P. mahaleb* (Jordano 1993) obtained in the Nava de las Correhuelas population to derive F_{IS} values, as suggested by Yeh & Boyle (1997). For each population we obtained the F_{IS} value multiplying the proportion of hermaphrodite trees in the population (functional female trees are obligately outcrossed) by the outcrossing value of S = 0.5268, determined experimentally (see Jordano 1993 for details).

A data matrix of individual × marker containing the band scoring information was transformed to allele frequencies under the assumption that each amplified band corresponds to a different RAPD locus. This dataset was used to calculate genetic diversity estimates, G_{ST} (Nei 1973) and genetic distances (Nei's unbiased distance estimate; Nei 1978) among populations and individual trees using the POPGENE (v. 1.31) software package of Yeh & Boyle (1997). The Nei's coefficient has been reported as being more robust to potential biases and artefacts in band scoring (Lamboy 1994). Despite limitations of RAPD data to assess population genetic statistics due to their dominant nature, we found robust consistency between F_{ST}, G_{ST} estimated for this dataset, and those based on the Shannon-Weaver index or assuming Hardy–Weinberg equilibrium (Chalmers *et al*. 1992; Bussell 1999). Genetic distance among individual trees in the studied populations was also estimated by the Euclidean metric of Excoffier *et al*. (1992), due to the assumption of an Euclidean distance definition among populations for the analysis of molecular variance (AMOVA) analyses.

We used the AMOVA procedure (Excoffier *et al*. 1992) to estimate the variance components of RAPD phenotypes associated to the geographical nested structure of the populations, with partitioning of variation in the genetic distance among individuals within populations, among populations within regions, and among regions. Regions were defined on the basis of similarity in elevation and location within the same mountain range. Analyses were performed with the ARLEQUIN package (Schneider *et al*. 1997); the analysis yields estimates of (Φ_{ST}, a shorthand for F_{ST}, for the overall comparison of populations and among pairs of populations (see also Stewart & Excoffier 1996). The significance was tested by resampling with $n = 10\,000$ randomizations. The distance matrix among populations based on Nei's (1978) unbiased distance estimate was used to construct unrooted phenetic trees with the program FITCH of PHYLIP package (Felsenstein 1989) using an average method (UPGMA).

The parameter F_{ST} has been frequently used o assess the patterns of population genetic subdivision expected under the 'island model' of population structure (Wright 1969), which assumes an infinite number of islands with the same effective size, without geographical structure, and exchanging a proportion of migrants per generation. However, for a finite number of islands (n-island model), Crow & Aoki (1984) proposed an analogous model where the multiallelic equivalent of F_{ST} is defined as:

$$G_{ST} \approx \frac{1}{4N_e ma + 1}$$

where $a = [n/(n-1)]^2$, and n is the number of subpopulations. We used the G_{ST} estimates to calculate the average number of immigrants per generation for each

locus ($Nm = (1 - G_{ST})/4G_{ST}$) and the mean value across loci (Slatkin & Barton 1989).

The relationships between the G_{ST} matrix and distance matrices (e.g. geographical distance, difference in elevation, phenological overlap in the period of ripe fruit availability) were estimated with the Mantel's test (Mantel 1967), using MANTEL program of the R package (Casgrain & Legendre 1998) with significance of the autocorrelation coefficient tested by resampling ($n = 10\,000$ randomizations). We used the multiple matrix extension of this test (Smouse *et al*. 1986) to assess the partial correlation between the genetic distance matrix and a second matrix, say geographical distance matrix, while holding the effects of a third matrix, say difference in elevation. The geographical distance matrix was estimated from the geographical coordinates of each population (DIST and LINKS programs of the R package, Casgrain & Legendre 1998). The elevation 'distance' matrix was constructed from the absolute difference in elevation among each pair of populations. The phenological overlap matrix was estimated from the phenological records at each population so that a score ranging between 0 and 1 was assigned to each pairwise comparison depending on the degree of phenological overlap of the fruiting phenophases. Thus, a 'phenological distance' index was estimated by $D_{ij} = \sqrt{1-S_{ij}}$, where S_{ij} is the phenological overlap or similarity and D_{ij} is bounded in the [0, 1] interval.

Results

We analysed a total of seven populations with 144 individual trees in total (Table 1). As most of these populations are small we sampled most of the individuals in them. The elevation range sampled (1300–1650 m) encompasses the altitudinal range where approximately 68% of the *Prunus mahaleb* populations in the study area are located (C.M. Herrera & P. Jordano, personal observation).

Four main regions can be identified for supra-population groupings of the seven populations listed in Table 1. Groups 1 and 2, the Roblehondo and Cañada de la Medianega populations, respectively, include the westernmost populations. The Calarilla, Nava de las Correhuelas, and Cabeza del Tejo populations are the three high-elevation sites (group 3); group 4 includes the mid-elevation populations of Nava Noguera, and Torcal del Cerecino, located at the eastern limit of the geographical area sampled.

The RAPD profile

A total of 12 primers, summarized in Table 2, generated 73 polymorphic amplification products in the range of 230–2550 bp. The average number of polymorphic markers across primers was 52.1%, ranging between 36.4% (OPD-16) and 80% (OPC-6).

Table 2 RAPD primers used in the survey of *Prunus mahaleb*, number of amplified products, and number of amplified products scored

Primer	Nucleotide sequence 5′ to 3′	Amplified fragments	Polymorphic amplified fragments
C4	CCGCATCTAC	12	5
C5	GATGACCGCC	13	8
C6	GAACGGACTC	10	8
C13	AAGCCTCGTC	16	7
C15	GACGGATCAG	11	6
C16	CACACTCCAG	12	7
D1	ACCGCGAAGG	12	6
D12	CACCGTATCC	10	6
D13	GGGGTGACGA	13	5
D15	CATCCGTGCT	11	5
D16	AGGGCGTAAG	11	4
D19	CTGGGGACTT	11	6

The percentage of polymorphic loci, considering those loci with the frequency of the most common allele ≤ 0.95, was relatively similar across populations, varying between 29.6% and 41.6%, with an average of 34.3% (Table 3). Presence of amplification products of OPD-13 (900 bp) and OPC-13 (3500 bp) were exclusive of trees from the Nava de las Correhuelas population while OPD13 (310 bp) was consistently absent in all trees. Trees from the Calarilla population exclusively had present the products for OPC-13 (1650 and 1800 bp), and OPD12 (600 bp) was consistently absent in all trees.

Variation among and within populations

Total genetic diversity varied between 0.1408 and 0.4998 (considering only polymorphic loci), with a mean (± 1SD) diversity of 0.1441 ± 0.0348. The mean Nei's genetic diversity for individual populations (h_i) ranged between 0.089 and 0.149, with mean value across populations of 0.1144 ± 0.0285 (mean ± 1SE; Table 3a). There was no obvious relationship between the gene diversity values and population size: the smallest value was recorded in the largest population (Nava Noguera), and the largest value also in a relatively large population (Torcal del Cerecino).

Mean G_{ST} value across loci was 0.1935, ranging between 0.0162 and 0.4685, and resulted in an average estimate for *Nm* of 1.191. Table 3b summarizes the genetic distance statistics (Nei's unbiased estimate; Nei 1978) for the studied populations. The genetic distance estimates range in the values of 0.0199–0.0538, with an average distance among populations of 0.0314. All pairwise distance estimates were significant ($P < 0.05$; $n = 5000$ permutations). Differences among populations when considering the multivariate space defined by the RAPD banding patterns can be

Table 3 (a) Patterns of genetic diversity for *Prunus mahaleb* populations; (b) and matrices of genetic distance (Nei 1978, unbiased estimate, G_{ST}; upper triangular) and *Nm* values (lower triangular). All localities are within Parque Natural de las Sierras de Cazorla, Segura y Las Villas, Jaén province, south-east Spain. *h* denotes mean Nei's gene diversity (±1 standard error), based on Nei's (1978) unbiased estimate; *H'*, Shannon's diversity index

(a) Genetic diversity

Population	% polymorphic loci	*h*	*H'*
1. Roblehondo	31.7	0.108 ± 0.059	0.1602
2. Cañada Medianega	30.3	0.104 ± 0.056	0.1571
3. Calarilla	36.6	0.119 ± 0.061	0.1819
4. Nava Noguera	29.6	0.089 ± 0.046	0.1374
5. Torcal del Cerecino	41.6	0.149 ± 0.066	0.2152
6. Cabeza del Tejo	33.1	0.115 ± 0.064	0.1749
7. Nava Correhuelas	37.3	0.117 ± 0.059	0.1775

(b) Genetic distance and *Nm* matrices

	1	2	3	4	5	6	7
1	—	0.0298	0.0367	0.0199	0.0242	0.0241	0.0320
2	1.0	—	0.0415	0.0317	0.0363	0.0355	0.0367
3	1.2	1.2	—	0.0474	0.0428	0.0345	0.0538
4	1.2	0.8	1.1	—	0.0262	0.0229	0.0305
5	1.6	0.9	1.6	1.0	—	0.0274	0.0332
6	1.1	0.8	1.0	0.7	1.3	—	0.0231
7	1.9	1.2	1.9	1.1	1.6	0.8	—

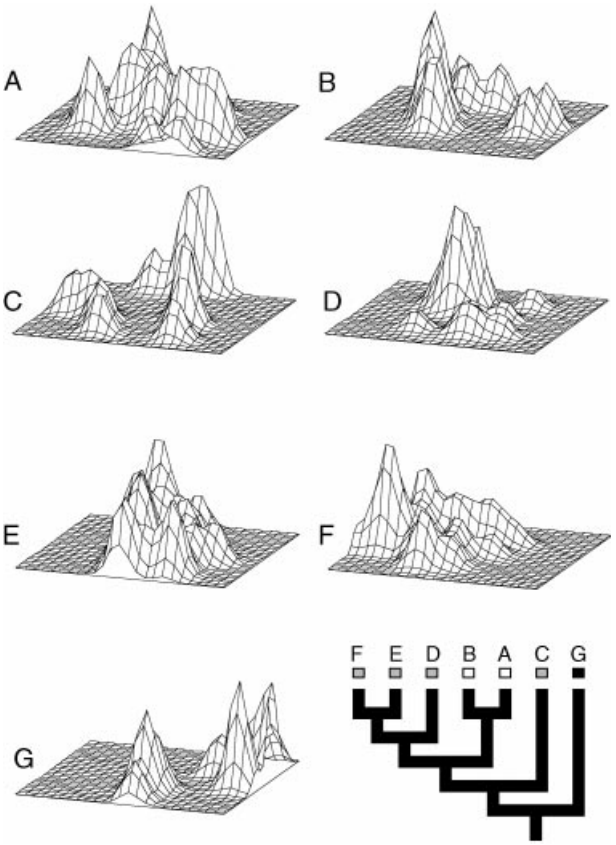

Fig. 1 Ordination of the individual tree scores on the two first axes of a principal coodinate analysis of RAPD patterns of seven *Prunus mahaleb* populations (A–G). The dataset consists of 144 trees and 142 RAPD loci. Each panel represents the scores for a distinct population, with the height of the three-dimensional surface proportional to the frequency of tree scores on the two axes. The phenogram (lower right panel) is based on Nei's (1978) unbiased genetic distance matrix among populations (Table 3), using an UPGMA algorithm. Different grey shades represent different elevation: blank, lower-elevation populations (< 1500 m); grey, 1500–1620 m; black, > 1620 m. See Table 1 for locality codes.

appreciated in the principal coordinate analysis (Fig. 1). The first two axes accounted for 53% of total variation. Each population has a distinct peak at different principal coordinate scores despite extensive overlap (Fig. 1). Moreover, the Calarilla, Nava de las Correhuelas, and Nava Noguera populations (Fig. 1 a,d,e) have a distinct unimodal distribution over the multivariate plane of the first two principal coordinates, while the Cañada de la Medianega, Cabeza del Tejo, Torcal del Cerecino, and Roblehondo populations (Fig. 1 b,c,f,g) have bimodal or multimodal peaks, suggesting variability in the genetic affinity of individual trees within populations. Populations in the first group are all in high-elevation sites, while the rest are all from lower elevation sites except Cabeza del Tejo.

The phenogram (Fig. 1) based on Nei's (1978) unbiased genetic distance matrix reveals a distinct grouping structure among populations. The populations at the lowest elevation sites (< 1500 m; Roblehondo and Torcal del Cerecino, Fig. 1 G,F) are separated from populations at mid-elevation sites (1500–1620 m, Calarilla, Nava de las Correhuelas, Nava Noguera; Fig. 1 A,B,E, respectively) and the Cabeza del Tejo population at the highest elevation (1635 m, Fig. 1C). The Cañada de la Medianega population (1540 m, Fig. 1B) also appears segregated from the rest, being the one more isolated geographically from the other mid-elevation sites.

Only 1.77% of variation in similarity of RAPD patterns was accounted for by differences among regions (Table 4), and this was not significant. However, both the fraction of variation accounted for by differences among populations (16.5%) and by differences among individuals within populations (81.8%) were significant (Table 4).

Correlates of population genetic variation

The matrix of G_{ST} values (Table 3) was significantly correlated with the matrix of Φ_{ST} pairwise distances (data

Source of variation	d.f.	Sum of squares	Variance component	%	*P*
Among regions	3	128.80	0.177	1.77	0.17
Among populations within regions	3	135.97	1.64	16.46	< 0.001
Among individuals within population	137	1119.93	8.17	81.77	< 0.001

Table 4 Results of the analysis of molecular variance (AMOVA) for 144 *Prunus mahaleb* individuals grouped in seven populations and four regions. The degrees of freedom (d.f.), sum of squares, variance components, and the fraction of total variation contributed by each nested component (%) and its associated significance (P; n = 10 000 permutations) are shown

not shown) obtained using AMOVA (r = 0.9989, P < 0.0001), and we present here the results using the G_{ST} matrix. The significant among-population structure revealed by the AMOVA analysis (Table 4) had only a marginal correlate with geographical distance. The Mantel's test between the G_{ST} matrix and the geographical distance matrix yielded r = 0.4169, P = 0.091, suggesting only marginal isolation by distance in these populations. Partialling out the effect of differences in elevation, resulted in an increase in significance, but still marginal (r = 0.4505, P = 0.072 for the matrix correlation between the G_{ST} matrix and the geographical distance matrix when controlling for the effects of the matrix of difference in elevation). The combined effect of both geographical and elevation distances (represented in a matrix with the sum of both distances) on the G_{ST} matrix was still only partially significant when controlling the regional distribution of the populations (r = 0.4197, P = 0.093). The trend is suggested by the lower *Nm* values among those population pairs that differ more in elevation (Fig. 2). Populations at similar elevations show the whole range of G_{ST} values (Fig. 2), while those populations differing in elevation ≥ 250 m consistently show low G_{ST} values.

The differences in elevation among populations entail a marked difference in phenology of both flowering and fruiting, increasing the delay in the phenophases when increasing elevation (Fig. 3). Thus, there was a significant matrix correlation between the matrix of elevation difference and the matrix of phenological difference (r = 0.6815, P = 0.007). Both the flowering and fruiting periods of *P. mahaleb* are relatively short, and the potential exists for reproductive isolation even between geographically close populations (e.g. Roblehondo and Calarilla) with a ≥ 250 m difference in elevation and nonoverlapping phenophases (Fig. 3). The G_{ST} matrix was only marginally correlated with the matrix of phenological 'distance' when controlling for the effect of elevation (r = 0.3043, P = 0.099) or geographical distances (r = 0.0506, P = 0.42). The analysis, therefore, reveals only marginal isolation of the *P. mahaleb* populations due to any type of distance, either geographical isolation, elevation isolation, or isolation by nonoverlapping flowering and fruiting phenophases. This is illustrated when assessing the correlations between the matrix of estimated *Nm* values among populations (Table 3b) and the matrices of geographical + elevation distance (r = −0.4519, P = 0.070), or phenological distance (r = −0.1129, P = 0.34). Thus, there is only a marginal trend for more isolated populations to exchange less immigrants (Fig. 2).

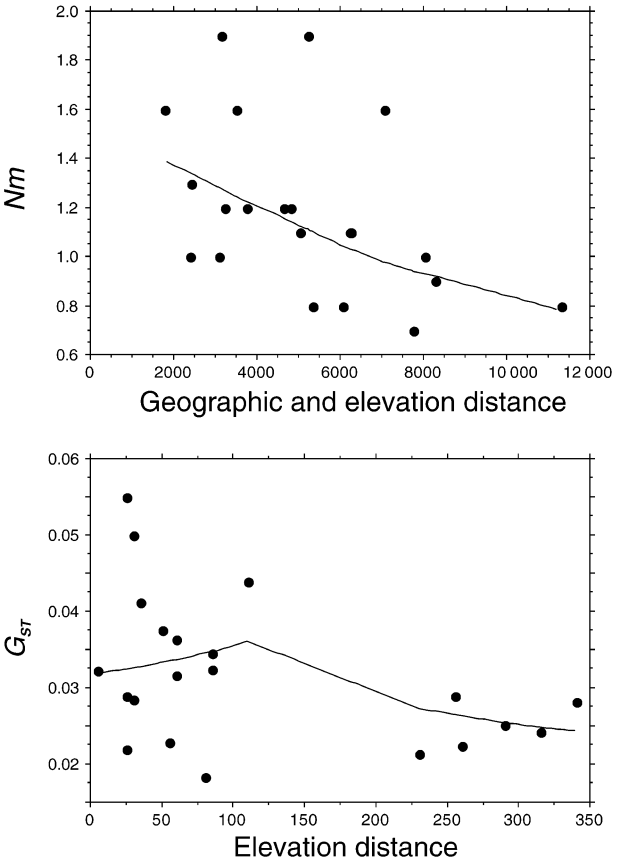

Fig. 2 (A) Relationship between the estimated number of immigrants per generation (*Nm*) and the geographical distance plus the difference in elevation between *Prunus mahaleb* populations. The difference in elevation was summed to the geographical distance value to obtain a 'pooled' estimate of spatial isolation. (B) Relationship between G_{ST} and the difference in elevation between pairs of populations. Thinlines represent LOWESS regression splines (Efron & Tibshirani 1991) to show the trend of covariation between variables. See text for results of randomization tests (Mantel's test) on these variables.

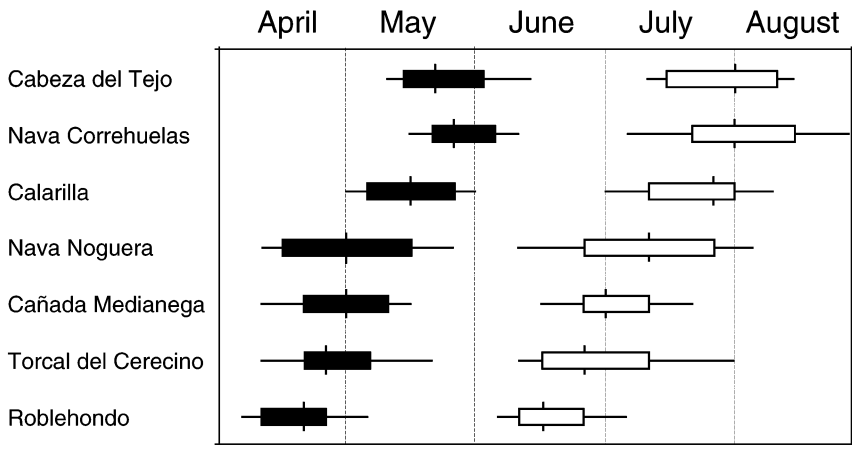

Fig. 3 Phenological overlap of *Prunus mahaleb* flowering and fruit ripening phenophases in the studied populations. Diagrams depict the length (horizontal line), interval dates when more than 50% of the individuals were in flower (filled bars) or with ripe fruits (blank bars), and dates with maximum number of individuals in flower or with ripe fruits (vertical lines).

Discussion

The studied populations of *Prunus mahaleb* occupy a relatively restricted area within the main distribution area of the species in south-eastern Spain which is characterized by a highly fragmented pattern of habitat occupancy (Hegi 1995). There, populations exist as isolated patches of individuals in mid- to high-elevation mountain areas with extensive tracts of forest without presence of the tree. Despite this situation of relative geographical isolation, low levels of population differentiation were found. Only 16.5% of variation in RAPD patterns was attributable to among-population differences, but this was a significant fraction of variation. At the spatial scale examined, relatively restricted to an area of ~100 km², this aggregate of populations exhibits high genetic homogeneity, with most genetic variation (81.8%) found within populations. Even the contribution of regional differences within this area (i.e. populations separated by watersheds or ridges) explained only 1.8% of variation. These findings are similar to those reported previously for *Prunus* spp. Dawson & Powell (1999), sampling a wider area for *P. africana*, reported only 23.2% for the among-population component in Cameroon and 22.5% for Madagascar. They reported that most variation in this *P. africana* sample was attributable to variation among countries (66.2%). Thus, it appears that *P. mahaleb* would exhibit a much larger among-population component of genetic variation when considering a wider geographical area, i.e. including populations from the Pyrenees, north-western Spain, the Alps, and other central European areas. Thus, Mariette *et al*. (1997) reported mean values of Nei's estimate of genetic diversity, based on allozymes, of 0.111, and G_{ST} of 0.052 for six wild *P. avium* populations in France, which are very similar to our estimate of Nei's index value (mean of 0.1144) but lower for G_{ST} (0.1935). Their lower estimate for G_{ST} most likely reflects higher fragmentation and reproductive isolation of south-east Spain populations.

Our AMOVA results with *P. mahaleb* indicate higher percentage of within-population variation than the average estimate (V_b = 71.42%) using RAPDs for endozoochorous species and well above the 49.2% reported for species with passive dispersal adaptations (Table 5). The G_{ST} values are within the ranges reported previously for allozyme variation in dicot woody species with mixed breeding system and endozoochorous seed dispersal (Hamrick *et al*. 1992; Hamrick *et al*. 1993; Gibson & Wheelwright 1995; Hamrick & Godt 1997), with average F_{ST} values for any combination of two traits ranging between F_{ST} = 0.099 (endozoochorous, long-lived perennials) and F_{ST} = 0.269 (endozoochorous species with mixed mating) (Hamrick & Godt 1997). These data are indicative of thorough seed dispersal across populations contributing to low genetic differentiation. Efficient seed dispersal for *P. mahaleb* is provided by a diverse assortment of frugivorous birds and mammals that usually remove and disperse a high fraction of the fruit crops every year (Jordano 1994; Jordano & Schupp 2000). The frugivore assemblage includes small-sized species (< 30 g body mass) like warblers, robins, and redstarts, which contribute to local seed dispersal, medium-sized birds like thrushes, corvids and wood pigeons, which typically show long seed delivery flights (Jordano & Schupp 2000), and carnivorous mammals like red foxes and stone martens with potentially large foraging ranges. A low G_{ST} value is, therefore, expected, but the estimate for *P. mahaleb* is well above figures for animal-dispersed tropical woody species (G_{ST} = 0.050; Loveless 1992). Spatial structuring of genetic diversity has been reported previously for fleshy-fruited species using RAPD markers (Graham *et al*. 1997; Bartish *et al*. 1999; but see Schierenbeck *et al*. 1997). Relatively high G_{ST} values and small *Nm* have been reported for bird-dispersed *Ocotea tenera* (Gibson & Wheelwright 1995) and explained by disproportionate postdispersal mortality in terms of recruitment limitation, without this being evidence against the possibility of long-distance delivery of seeds by the frugivores. A similar

Table 5 Patterns of genetic variability within populations of endozoochorous plant species, estimated from RAPD patterns with the AMOVA procedure. Entries for anemochorous and dyszoochorous tree species and species with other means of seed dispersal (autochorous, barochorous, etc.) are given as summarized values taken mostly from Bartish *et al*. (1999) and Bussell (1999)

Species	No. of populations	No. plants / population	No. markers	% within population variation	Nei's unbiased genetic distance*	Reference
Endozoochorous trees and shrubs						
Prunus mahaleb	7	10–32	142	81.8	0.019–0.054	This study
Hippophae r. rhamnoides	10	9–14	156	85.0	0.011–0.084	Bartish *et al*. (1999)
Lonicera periclymenum	2	115	43	84.1	—	Grashof-Bokdam *et al*. (1998)
H. rhamnoides mongolica	1	18	156	—	0.092–0.151	Bartish *et al*. (1999)
Acorus gramineus	17	15	34	36.0	—	Schierenbeck *et al*. (1997)
Dendropanax arboreus	5	4–21	29	97.0	—	Schierenbeck *et al*. (1997)
Protium glabrum	3	15–19	20	100	—	Schierenbeck *et al*. (1997)
Prunus africana	10	4–10	48	76.8, 77.5†	0.020–0.137	Dawson & Powell (1999)
Iliama corei	2	15	99	45.4	—	Stewart *et al*. (1996)
Iliama remota	3	19	7	45.4	—	Stewart *et al*. (1996)
Amelanchier bartramiana	—	18–29	77	—	—	Campbell *et al*. (1999)
Amelanchier laevis	—	14–28	76	—	—	Campbell *et al*. (1999)
Vaccinium macrocarpon	6	4	90	66.9	—	Stewart & Excoffier (1996)
Anemochorous and dyszoochorous trees	—	—	—	82.0 ± 12.2 (13)	0.243 ± 0.091 (5)	Bussell (1999)
Other types	—	—	—	49.2 ± 26.4 (12)	0.253 ± 0.194 (10)	Bussell (1999), Bartish *et al*. (1999)

* Range of values for pairwise comparisons among populations.
†Two estimates given for populations from two geographical areas.

interpretation can be advanced for *P. mahaleb*, although we also found significant genetic structuring suggesting isolation by distance.

Our results with the AMOVA analysis and the correlates of G_{ST} and Nm matrices with those measuring different levels of population spatial isolation are indicative of significant structuring of the genetic variation at the spatial scales examined. First, we found a significant among-population component of variation in RAPD pattern, although relatively small. Second, we obtained marginally significant trends for both G_{ST} and Nm to decrease with increasing geographical distance and increasing temporal segregation of flowering and fruiting phenophases due to elevation differences. For insect-pollinated and animal-dispersed species such as *P. mahaleb*, temporal segregation of reproductive phenophases would be an important component of reproductive isolation. First, insect-mediated pollen flow can reach potentially long distances (Stacy *et al*. 1996; Nason *et al*. 1998) whenever reproductive individuals in anthesis are coincident temporally, and more detailed analysis is required to separate the contributions of pollen- and seed-mediated gene flow to population differentaition (Ennos 1994; McCauley 1997). For species like *Prunus*, typically showing very short anthesis periods at the individual level (Guitián *et al*. 1993; Jordano 1993), noncoincident flowering can severely limit the possibilities of gene flow among populations. During the fruiting phenophase the situation is similar. Avian frugivores feeding on *P. mahaleb* fruits, specially medium and large sized birds, can move in flocks foraging in different tree clumps or populations, even within the same day. For example, 77.5% of the exit flights recorded for birds feeding at the trees were to perches within 30 m of the feeding tree and only 18.5% were to perches > 15 m away of any *P. mahaleb* tree. But 60.2% of the exit flights by mistle thrushes were to perches located > 30 m away from the feeding tree, not infrequently > 100 m away (Jordano & Schupp 2000). Observational evidence (P. Jordano, personal observation) indicates that mistle thrush flocks can track the availability of feeding trees in different patches and visit preferentially those localities with fruit available at the same time (also see Wheelwright 1983; Sallabanks 1993). Additional study, currently underway, integrating medium- and long-scale patterns of frugivore movements with estimates of seed-mediated gene flow within and among populations, using microsatellite markers, will help us to understand the occurrence of significant genetic structuring and high values of within-population genetic diversity.

There is evidence that establishment of fleshy-fruited species with adaptations to endozoochorous seed dispersal

could be more limited by recruitment than by availability of seeds or seed delivery (i.e. recruitment vs. dispersal limitation; Clark *et al*. 1999). Growing evidence, based on molecular markers, supports this view by providing evidence of low levels of population differentiation among animal-dispersed woody species when compared to species with other life-history syndromes (Bartish *et al*. 1999; Bussell 1999; present study; also see Hamrick *et al*. 1993 for evidence based on allozyme markers). Thorough removal of literally thousands of *P. mahaleb* fruits would be expected to result in extensive delivery of seeds throughout all available patches, yet seed rain is extremely nonrandom and clumped (Jordano & Schupp 2000). A situation of isolation by distance, as pointed out by our analysis of population differentiation, in such a scenario would be driven primarily by vicariant factors not directly related to gene flow patterns (Bossart & Prowell 1998). First, demographic bottlenecks acting during the recruitment stages that follow seed dispersal cause disproportionately high mortality of dispersed seeds, established seedlings, and saplings (Schupp 1990; Jordano & Herrera 1995; Schupp & Fuentes 1995; Schupp 1995), and this is also the case for *P. mahaleb* (Schupp 1993; Schupp & Fuentes 1995; Schupp & Jordano, in prep.). Thus, low estimates of *Nm* would be indicative of strong recruitment limitation, not restricted frugivore-mediated seed dispersal (see also Gibson & Wheelwright 1995). Second, recruitment limitation simply adds delays to the establishment of reproductive adults (Epperson & Alvarez-Buylla 1997; Mariette *et al*. 1997), with the net result that, despite extensive seed dispersal by efficient frugivores, the genetic make-up of the reproductive population represents only a fraction of the total genetic diversity across all the demographic stages (seeds, seedlings, saplings). Moreover, for outcrossed, insect- or wind-pollinated and animal-dispersed species, high within-population genetic diversity would be maintained chiefly by a transient, diffuse genetic structuring with substantial build-up over repeated generations of the life cycle (Epperson & Alvarez-Buylla 1997).

Theory predicts that even very low migration rates would be enough to counterbalance local differentiation among populations if they are at drift/migration equilibrium (Kimura 1983), yet most temperate tree populations are probably away from equilibrium since glacial periods. The patterns documented for *P. mahaleb* are, therefore, interpretable as a combination of efficient dispersal of seeds by frugivores contributing to high within-population genetic diversity and a process of isolation by distance related to highly fragmented populations with frequent recruitment limitation. Future analysis of population patterns of differentiation and gene flow should bridge evidences from both molecular methods and demographic studies.

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References

Badenes ML, Parfitt DE (1995) Phylogenetic relationships of cultivated *Prunus* species from an analysis of chloroplast DNA variation. *Theoretical and Applied Genetics*, **90**, 1035–1041.

Bartish IV, Jeppsson N, Nybom H (1999) Population genetic structure in the dioecious pioneer plant species *Hippophae rhamnoides* investigated by random amplified polymorphic DNA (RAPD) markers. *Molecular Ecology*, **8**, 791–802.

Bossart JL, Prowell DP (1998) Genetic estimates of population structure and gene flow: limitations, lessons and new directions. *Trends in Ecology and Evolution*, **13**, 202–206.

Bussell JD (1999) The distribution of random amplified polymorphic DNA (RAPD) diversity amongst populations of *Isotoma petraea* (Lobeliaceae). *Molecular Ecology*, **8**, 775–789.

Campbell CS, Alice LA, Wright WA (1999) Comparisons of within-population genetic variation in sexual and agamospermous *Amelanchier* (Rosaceae) using RAPD markers. *Plant Systematics and Evolution*, **215**, 157–167.

Casas AM, Igartua E, Balaguer G, Moreno MA (1999) Genetic diversity of *Prunus* rootstocks analyzed by RAPD markers. *Euphytica*, **110**, 139–149.

Casgrain P, Legendre P (1998) *The R package for multivariate and spatial analysis, v. 4.0*. Available at: http://www.fas.umontreal.ca/BIOL/Casgrain/R/index.html. University of Montreal, Montreal, Canada.

Chalmers KJ, Waugh R, Spent JI, Simmons AJ, Powell W (1992) Detection of genetic variation between and within populations of *Gliricidia sepium* and *G. maculata* using RAPD markers. *Heredity*, **69**, 465–472.

Chase MR, Moller C, Bawa KS (1996) Distant gene flow in tropical trees. *Nature*, **383**, 398–399.

Cheung WY, Hubert N, Landry BS (1993) A simple and rapid DNA microextraction method for plant, animal, and insect suitable for RAPD and PCR analyses. *PCR Methods and Applications*, **3**, 69–70.

Clark JS, Beckage B, Camill P *et al*. (1999) Interpreting recruitment limitation in forests. *American Journal of Botany*, **86**, 1–16.

Cobb B (1997) Optimization of RAPD fingerprinting. In: *Fingerprinting Methods Based on Arbitrarily Primed PCR* (eds Micheli MR, Bova R), pp. 93–102. Springer-Verlag, Berlin.

Crow JF, Aoki K (1984) Group selection of a polygenic behavioral trait: estimating the degree of population subdivision. *Proceedings of the National Academy of Sciences of the USA*, **81**, 6073–6077.

Dawson IK, Powell W (1999) Genetic variation in the afromontane tree *Prunus africana*, an endangered medicinal species. *Molecular Ecology*, **8**, 151–156.

Efron B, Tibshirani R (1991) Statistical data analysis in the computer age. *Science*, **253**, 390–395.

Ennos RA (1994) Estimating the relative rates of pollen and seed migration among plant populations. *Heredity*, **72**, 250–259.

Epperson BK, Alvarez-Buylla ER (1997) Limited seed dispersal and genetic structure in life stages of *Cecropia obtusifolia*. *Evolution*, **51**, 275–282.

Eriksson O, Bremer B (1992) Pollination systems, dispersal modes, life forms, and diversification rates in angiosperm families. *Evolution*, **46**, 258–266.

Excoffier L, Smouse PE, Quattro JM (1992) Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics*, **131**, 479–491.

Felsenstein J (1989) PHYLIP — Phylogeny Inference Package (Version 3.2). *Cladistics*, **5**, 164–166.

Fragoso JMV (1997) Tapir-generated seed shadows: scale-dependent patchiness in the Amazon rain forest. *Journal of Ecology*, **85**, 519–529.

Fritsch P, Rieseberg LH (1996) The use of random amplified polymorphic DNA (RAPD) in conservation genetics. In: *Molecular Genetic Approaches in Conservation* (eds Smith TB, Wayne RK), pp. 54–73. Oxford University Press, New York.

Gibson JP, Wheelwright NT (1995) Genetic structure in a population of a tropical tree *Ocotea tenera* (Lauraceae): influence of avian seed dispersal. *Oecologia*, **103**, 49–54.

Graham J, Squire GR, Marshall B, Harrison RE (1997) Spatially dependent genetic diversity within and between colonies of wild raspberry *Rubus idaeus* detected using RAPD markers. *Molecular Ecology*, **6**, 1001–1008.

Grashof-Bokdam CJ, Jansen J, Smulders MJM (1998) Dispersal patterns of *Lonicera periclymenum* determined by genetic analysis. *Molecular Ecology*, **7**, 165–174.

Guitián J, Guitián P, Sánchez JM (1993) Reproductive biology of two *Prunus* species (Rosaceae) in the Northwest Iberian Peninsula. *Plant Systematics and Evolution*, **185**, 153–165.

Hamrick JL, Godt MJ, Sherman-Boyles SL (1992) Factors influencing levels of genetic diversity in woody plant species. *New Forests*, **6**, 95–124.

Hamrick JL, Godt MJW (1997) Effects of life history traits on genetic diversity in plant species. In: *Plant Life Histories. Ecology, Phylogeny and Evolution* (eds Silvertown J, Franco M, Harper JL), pp. 102–118. Cambridge University Press, Cambridge, UK.

Hamrick JL, Murawski DA, Nason JD (1993) The influence of seed dispersal mechanisms on the genetic structure of tropical tree populations. In: *Frugivory and Seed Dispersal: Ecological and Evolutionary Aspects* (eds Fleming TH, Estrada A), pp. 281–297. Kluwer Academic Publisher, Dordrecht, The Netherlands.

Hegi G (1995) *Illustrierte Flora Von Mittleeuropa*. Blackwell Wissenschaft, Berlin, Germany.

Heinkel R, Hartmann W, Stosser R (1998) Bestimmung der Befruchtungsrate mit Hilfe der RAPD-Technik nach Bestäubung mit verschiedenen Pollenspendern bei *Prunus* x *domestica*, cv. 'Ortenauer'. *Journal of Applied Botany*, **72**, 174–179.

Herrera CM (1989) Frugivory and seed dispersal by carnivorous mammals, and associated fruit characteristics, in undisturbed mediterranean habitats. *Oikos*, **55**, 250–262.

Herrera CM, Jordano P (1981) *Prunus mahaleb* and birds: the high efficiency seed dispersal system of a temperate fruiting tree. *Ecological Monographs*, **51**, 203–221.

Herrera CM, Jordano P, López Soria L, Amat JA (1994) Recruitment of a mast-fruiting, bird-dispersed tree: bridging frugivore activity and seedling establishment. *Ecological Monographs*, **64**, 315–344.

Heun M, Helentjaris T (1993) Inheritance of RAPDs in F1 hybrids of corn. *Theoretical and Applied Genetics*, **85**, 961–968.

Howe HF, Schupp EW, Westley LC (1985) Early consequences of seed dispersal for a neotropical tree (*Virola surinamensis*). *Ecology*, **66**, 781–791.

Huff DR, Peakall R, Smouse PE (1993) RAPD variation within and among natural populations of outcrossing buffalograss (*Buchloe dactyloides*). *Theoretical and Applied Genetics*, **86**, 927–934.

Jordano P (1992) Fruits and frugivory. In: *Seeds: the Ecology of Regeneration in Plant Communities* (ed. Fenner M), pp. 105–156. Commonwealth Agricultural Bureau International, Wallingford, England.

Jordano P (1993) Pollination biology of *Prunus mahaleb* L. deferred consequences of gender variation for fecundity and seed size. *Biological Journal of the Linnean Society*, **50**, 65–84.

Jordano P (1994) Spatial and temporal variation in the avian-frugivore assemblage of *Prunus mahaleb*: patterns and consequences. *Oikos*, **71**, 479–491.

Jordano P (1995) Frugivore-mediated selection on fruit and seed size: birds and St. Lucie's cherry, *Prunus mahaleb*. *Ecology*, **76**, 2627–2639.

Jordano P, Herrera CM (1995) Shuffling the offspring: uncoupling and spatial discordance of multiple stages in vertebrate seed dispersal. *Ecoscience*, **2**, 230–237.

Jordano P, Schupp EW (2000) Determinants of seed dispersal effectiveness: the quantity component in the *Prunus mahaleb*–frugivorous bird interaction. *Ecological Monographs*, 70.

Julliot C (1997) Impact of seed dispersal of red howler monkeys *Alouatta seniculus* on the seedling population in the understorey of tropical rain forest. *Journal of Ecology*, **85**, 431–440.

Kimura M (1983) *The Neutral Theory of Molecular Evolution*. Cambridge University Press, Cambridge, UK.

Lamboy WF (1994) Computing genetic similarity coefficients from RAPD data: the effects of PCR artifacts. *PCR Methods and Applications*, **4**, 31–37.

Loiselle B, Sork VL, Nason J, Graham C (1995a) Spatial genetic structure of a tropical understory shrub, *Psychotria officinalis* (Rubiaceae). *American Journal of Botany*, **82**, 14209–11425.

Loiselle BA, Sork VL, Graham C (1995b) Comparison of genetic variation in bird-dispersed shrubs of a tropical wet forest. *Biotropica*, **27**, 487–494.

Loveless MD (1992) Isozyme variation in tropical trees: patterns of genetic organization. *New Forests*, **6**, 67–94.

Lynch M, Milligan G (1994) Analysis of population genetic structure with RAPD markers. *Molecular Ecology*, **3**, 91–99.

McCauley DE (1997) The relative contributions of seed and pollen movement to the local genetic structure of *Silene alba*. *Journal of Heredity*, **88**, 257–263.

Mantel N (1967) The detection of disease clustering and a generalized regression approach. *Cancer Research*, **27**, 209–220.

Mariette S, Lefranc M, Legrand P, Taneyhill D, Frascaria-Lacoste N, Machon N (1997) Genetic variability in wild cherry populations in France. Effects of colonizing processes. *Theoretical and Applied Genetics*, **94**, 904–908.

Nason JD, Hamrick JL (1997) Reproductive and genetic consequences of forest fragmentation: two case studies of neotropical canopy species. *Journal of Heredity*, **88**, 264–276.

Nason JD, Herre EA, Hamrick JL (1996) Paternity analysis of the breeding structure of strangler fig populations: evidence for substantial long-distance wasp dispersal. *Journal of Biogeography*, **23**, 501–512.

Nason JD, Herre EA, Hamrick JL (1998) The breeding structure of a tropical keystone plant resource. *Nature*, **391**, 685–687.

Nei M (1973) Analysis of gene frequencies in subdivided populations. *Proceedings of the National Academy of Sciences of the USA*, **70**, 3321–3323.

Nei M (1978) Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics*, **89**, 583–590.

Ouborg NJ, Piquot Y, van Groenendael JM (1999) Population genetics, molecular markers and the study of dispersal in plants. *Journal of Ecology*, **87**, 551–568.

Palacios C, González-Candelas F (1997) Analysis of population genetic structure and variability using RAPD markers in the endemic and endangered *Limonium dufourii* (Plumbaginaceae). *Molecular Ecology*, **6**, 1107–1121.

Sallabanks R (1993) Hierarchical mechanisms of fruit selection by an avian frugivore. *Ecology*, **74**, 1326–1336.

Schierenbeck KA, Skupski M, Lieberman D, Lieberman M (1997) Population structure and genetic diversity in four tropical tree species in Costa Rica. *Molecular Ecology*, **6**, 137–144.

Schnabel A, Nason JD, Hamrick JL (1998) Understanding the population genetic structure of *Gleditsia triacanthos* L. seed dispersal and variation in female reproductive success. *Molecular Ecology*, **7**, 819–832.

Schneider S, Kneffer JM, Roessli D, Excoffier L (1997) *ARLEQUIN, vers. 1.1: a software for population genetic data analysis*. Genetics and Biometry Laboratory, University of Geneva, Switzerland.

Schupp EW (1990) Annual variation in seedfall, postdispersal seed predation, and recruitment of a neotropical tree. *Ecology*, **71**, 504–515.

Schupp EW (1993) Quantity, quality, and the efectiveness of seed dispersal by animals. In: *Frugivory and Seed Dispersal: Ecological and Evolutionary Aspects* (eds Fleming TH, Estrada A), pp. 15–29. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Schupp EW (1995) Seed-seedling conflicts, habitat choice, and patterns of plant recruitment. *American Journal of Botany*, **82**, 399–409.

Schupp EW, Fuentes M (1995) Spatial patterns of seed dispersal and the unification of plant population ecology. *Écoscience*, **2**, 267–275.

Slatkin M, Barton NH (1989) A comparison of three indirect methods for estimating average levels of gene flow. *Evolution*, **43**, 1349–1368.

Smouse PE, Long JC, Sokal RR (1986) Multiple regression and correlation extensions of the Mantel test of matrix correspondence. *Systematic Zoology*, **35**, 627–632.

Sork VL, Campbell DR, Dyer R *et al.* (1998) *Gene flow in fragmented, managed, and continuous populations. Research paper no. 3*. Available at: <http:www.nceas.ucsb.edu/papers/geneflow>. National Center for Ecological Analysis and Synthesis, Santa Barbara, California, USA.

Sork VL, Nason J, Campbell DR, Fernández JF (1999) Landscape approaches to historical and contemporary gene flow in plants. *Trends in Ecology and Evolution*, **14**, 224.

Stacy EA, Hamrick JL, Nason JD, Hubbell SP, Foster RB, Condit R (1996) Pollen dispersal in low-density populations of three neotropical tree species. *American Naturalist*, **148**, 275–298.

Stewart CN, Excoffier L (1996) Assessing population genetic structure and variability with RAPD data: application to *Vaccinum macrocarpon* (American cranberry). *Journal of Evolutionary Biology*, **9**, 153–171.

Stewart CN, Rosson G, Shirley BW, Porter DM (1996) Population genetic variation in rare and endangered Iliamna (Malvaceae) in Virginia. *Biological Journal of the Linnean Society*, **58**, 357–369.

Stiles FG (1975) Ecology, flowering phenology, and hummingbird pollination of some Costa Rican *Heliconia* species. *Ecology*, **56**, 285–301.

Valle F, Gómez F, Mota F, Díaz C (1989) *Parque Natural de Cazorla, Segura y Las Villas. Guía botánico-ecológica*. Editorial Rueda, Madrid.

Wang D, Karle R, Brettin TS, Iezzoni AF (1998) Genetic linkage map in sour cherry using RFLP markers. *Theoretical and Applied Genetics*, **97**, 1217–1224.

Warburton ML, Bliss FA (1996) Genetic diversity in peach (*Prunus persica* L. Batch) revealed by randomly amplified polymorphic DNA (RAPD) markers and compared to inbreeding coefficients. *Journal of the American Society of Horticultural Sciences*, **121**, 1012–1019.

Webb DA (1968) *Prunus* L. In: *Flora Europaea, Vol. 2* (eds Tutin TG, Heywood VH, Burges NA *et al.*), pp. 77–80. Cambridge University Press, Cambridge, England.

Wheelwright NT (1983) Fruits and the ecology of resplendent quetzals. *Auk*, **100**, 286–301.

Wright S (1969) *Evolution and the Genetics of Populations, Vol. 2: the Theory of Gene Frequencies*. University of Chicago Press, Chicago, USA.

Yeh FC, Boyle TJB (1997) Population genetic analysis of co-dominant and dominant markers and quantitative traits. *Belgian Journal of Botany*, **129**, 157.

These results are part of a broader study on the effect of animal frugivores on the genetics and demography of fleshy-fruited plants. Ongoing research includes the use of microsatellite markers in parentage analysis to study seed dispersal in relation to frugivore foraging. P. Jordano is interested in the evolutionary ecology of plant-animal mutualisms and the demographic and genetic effects of the interaction. J. A. Godoy is interested in the development and application of molecular techniques to studies of ecology and conservation of endangered vertebrate and plant species (molecular sexing, individual, parentage, and population genetic analyses). They coordinate the Molecular Ecology Laboratory at the Estación Biológica de Doñana, CSIC.
