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A GENETIC ANALYSIS OF HYDROLOGICALLY DISPERSED SEEDS OF *HIBISCUS MOSCHEUTOS* (MALVACEAE)[1]

HIROSHI KUDOH[2,3,4] AND DENNIS F. WHIGHAM[3]

[2]Department of Biological Sciences, Graduate School of Science, Tokyo Metropolitan University, Minami-Osawa 1-1, Hachioji 192-0397, Japan; and [3]Smithsonian Environmental Research Center, Box 28, Edgewater, Maryland 21037 USA

The dispersal of floating seeds in wetland habitats should influence the genetic characteristics of plant metapopulations. We examined gene flow of a hydrochorous wetland macrophyte, *Hibiscus moscheutos* L. (Malvaceae), by analyzing allozyme variation in current-year floating-seed populations. The genetic composition of floating seeds was compared to the genetic composition of established populations of *H. moscheutos* that had been previously analyzed in the same areas. The *F* statistics demonstrated that genetic structuring among floating-seed populations was weak or absent, indicating that seeds from source populations were thoroughly mixed. Floating-seed populations had an excess of homozygotes, a different situation than had previously been found in established populations. The exchange of seeds was greatest among *H. moscheutos* populations that were adjacent to a tidal stream. We conclude that populations adjacent to the tidal streams are part of a metapopulation that serves as a reserve of genetic variation in the system. Although established populations of *H. moscheutos* that are not close to the tidal stream are relatively isolated genetically, we found evidence that they also contribute to the floating-seed populations within the estuary.

Key words: allozyme variation; gene flow; *Hibiscus moscheutos*; hydrochory; Malvaceae; metapopulation approach; seed dispersal; wetland plants.

In tidal and nontidal freshwater and brackish wetlands, interactions between topography and hydrology determine the depth, timing and duration of flooding, the length of the dry or exposed period, and patterns of salinity in estuarine environments. Microtopographic variability in such wetlands also results in a diversity of habitats with ranges of environmental conditions within short distances (Reimold, 1977; Whigham et al., 1978; Simpson et al., 1983). These spatial discontinuities in physical environments have a striking influence on the distribution of plant species in wetland habitats (Simpson et al., 1983; Leck and Simpson, 1994; Silvertown et al., 1999). Wetland vegetation usually exhibits marked zonation or patchiness in species distributions, and patchily distributed populations are vulnerable to size fluctuations or extinction due to environmental variation and demographic stochasticity (Simpson et al., 1983; Leck and Simpson, 1994, 1995; Husband and Barrett, 1998). As a result, the dynamics and persistence of patchily distributed plant species may depend on the existence of an array of interconnected populations (metapopulation), which are linked by the movement of genes among local populations (reviewed in Husband and Barrett, 1996; Giles and Goudet, 1997). Recently, ecologists have become increasingly interested in dispersal among populations that takes place across heterogeneous landscapes (Olivieri and Gouyon, 1997; Sork et al., 1999).

Hydrochory (water dispersal) is a common method of seed dispersal among wetland plant species (Waser, Vickery, and Price, 1982; Schneider and Sharitz, 1988; Leck, 1989; Edwards, Wyatt, and Sharitz, 1994; Cellot, Mouillot, and Henry, 1998; Middleton, 1999), and effective seed dispersal over hundreds of metres or kilometres has been demonstrated (Nilsson, Gardfjell, and Grelsson, 1991; Lonsdale, 1993). Water dispersal is also a mechanism for genetic exchange among local populations within a metapopulation. Several genetic studies of hydrochorous species have demonstrated effective long-distance seed exchanges among populations in aquatic habitats (Kudoh and Whigham, 1997; Akimoto, Shimamoto, and Morishima, 1998; Gornall, Hollingsworth, and Preston, 1998; Schlueter and Guttman, 1998). The pattern of gene exchange among local populations of wetland species should, however, be influenced by factors such as the degree of hydrological connectivity between populations and the density of wetland vegetation and litter.

The distribution of genetic variation within and among plant populations has been used, for example, as a method to evaluate the combined effects of breeding systems and dispersal of pollen and seeds (Brown, 1979; Loveless and Hamrick, 1988; Hamrick, 1989). Genetic studies of extant populations often represent the only information to assess migration among multiple populations, and they provide estimates of gene flow averaged over long times, or historical gene flow (Slatkin, 1987). They may not necessarily estimate contemporary gene flow on an appropriate ecological time scale.

In a previous study (Kudoh and Whigham, 1997), we investigated historical gene flow among populations of *Hibiscus moscheutos* L. (Malvaceae), a hydrochorous wetland macrophyte, in an estuarine system and adjacent nontidal freshwater wetland using allozyme polymorphism. We found that populations of *H. moscheutos* that were adjacent to the tidal stream shared a higher degree of genetic relatedness than populations that were not adjacent to the tidal stream or were in upstream nontidal freshwater wetlands. We inferred that the observed genetic patterns could be explained by patterns of water dispersal of seeds and not by the exchange of pollen among populations (Kudoh and Whigham, 1997). In this study, we collected floating seeds from three locations within the intertidal habitat studied earlier (Kudoh and Whigham, 1997). We estimated the genetic contribution of the ten previously studied

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[4] Author for correspondence.

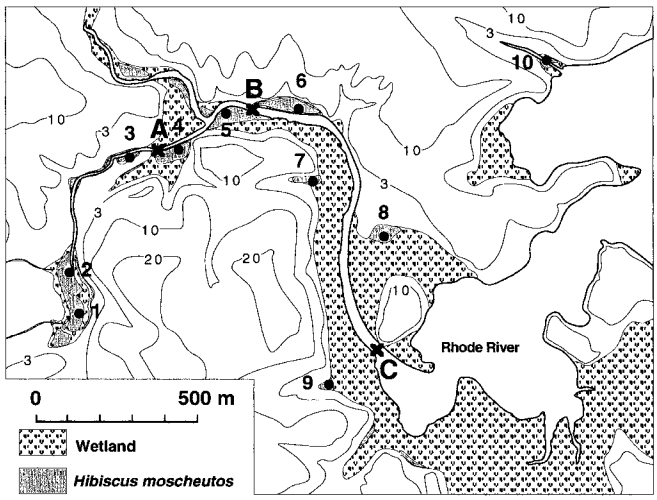

Fig. 1. Map showing the distribution of *Hibiscus moscheutos* in the research area and the locations of three sites (A, B, and C) where floating seeds were collected. Locations of ten sampling sites (1–10) from mature plants in the previous study (Kudoh and Whigham, 1997) are also shown. Contours in the map indicate elevation above sea level (m). The hydrologic relationships between the ten *H. moscheutos* populations (1–10) and the three floating-seed sampling sites (A–C) are described in the Materials and methods section.

H. moscheutos populations to the floating-seed populations. Comparison of the two data sets allowed us to evaluate our earlier conclusion about the importance of water dispersal of *H. moscheutos* seeds. Comparison of the two data sets also allowed us to evaluate the role of long-distance dispersal in preserving rare genotypes in the system.

MATERIALS AND METHODS

Plants—*Hibiscus moscheutos* is native to freshwater and brackish wetlands in the eastern United States (Blanchard, 1976; Beal, 1977; Brown and Brown, 1984; Cahoon and Stevenson, 1986; Spira, 1989). In Maryland, wetlands dominated by *H. moscheutos* cover 4.9 and 0.2% of the freshwater and brackish wetland habitats, respectively (McCormick and Somes, 1982). *Hibiscus moscheutos* is a long-lived perennial; older plants may form compact multistemmed clumps, but they are not rhizomatous or stoloniferous and cannot spread away from the point of establishment except by seed dispersal. The flowers are large (10–15 cm across) and showy, with one or two open flowers per shoot each day during the flowering period. The flowering period extends from late July to early September. Fruit maturation takes 3–4 wk, and most seeds are released from dehisced fruits in October and November. The seeds are thick-coated and buoyant (Blanchard, 1976), thus it is possible for seeds to be dispersed by water after they have fallen from the fruits.

Sampling—Study sites were located within a 1.5 km^2 area along a fresh to brackish water gradient in the Rhode River subestuary (38°53′ N, 76°33′ W) of Chesapeake Bay (Fig. 1). Ten *H. moscheutos* populations along a nontidal to tidal gradient along Muddy Creek (sites 1–10; Fig. 1) were sampled in our previous study on allozyme variation in established populations (Kudoh and Whigham, 1997). Sites 1 and 2 are in a freshwater nontidal wetland, locally known as Mill Swamp (Fig. 1). Seeds produced by *H. moscheutos* populations in Mill Swamp are potentially dispersed to sites 3–10 if they float out of the wetland through the stream that connects sites 1 and 2 to the intertidal zone. The portion of the intertidal zone that connects the Rhode River estuary to Mill Swamp is locally known as Muddy Creek. Muddy Creek is shown in Fig. 1 as the stream that connects site 2 to sampling site C. Sites 3–9 are located along the Muddy Creek portion of the intertidal zone and all are hydrologically connected by tides. Site 10 is the only site that is located outside of the Muddy Creek portion of the estuary, but it is hydrologically connected to sites 3–9 by incoming and outgoing tides. Site 10 is an intertidal wetland and seeds that are produced at the site potentially can reach sites 3–9. *Saururus cernuus, Polygonum arifolium, Polygonum punctatum,* and *Leersia oryzoides* were the most common emergent macrophyte species in Mill Swamp. At the brackish sites, *Typha angustifolia, Spartina cynosuroides, Scirpus olneyi,* and *Polygonum punctatum* were the most commonly associated species. We do not know the age of the extant populations nor do we know how often seedlings are recruited into these *H. moscheutos* populations. Long-term observations of *H. moscheutos* in the Rhode River estuary, however, indicate the existing populations are very old and that they have changed very little over the past 25 yr (D. F. Whigham, personal observation). Seedling mortality appears to be high following germination, and recruitment seems to be limited mostly to sites impacted by muskrat activity (e.g., muskrat feeding stations and lodges) (H. Kudoh and D. F. Whigham, unpublished data). For more detailed information of these ten sites, see Kudoh and Whigham (1997).

We collected floating seeds from three locations on Muddy Creek (sites A, B, and C; Fig. 1) in late November 1996, after most seeds had fallen from dehisced fruits. Site A was near the limit of tidal influence in the estuary and was chosen to be near sites 1 and 2, which represent potential sources of seeds from a nontidal freshwater habitat. Site C was chosen because it is ''downstream'' of all but one of the populations sampled by Kudoh and Whigham (1997). Site B was chosen to represent an intermediate location between sites A and C. We sampled seeds floating near the center of the stream with a hand net extended from a canoe. Sampling occurred during daytime, for ~1 h per site for each sampling day. Sampling at each site was done over five consecutive days (i.e., approximately ten tide cycles), until we obtained enough seeds for allozyme analyses. Forty-two, 110, and 100 viable seeds were collected at sites A, B, and C, respectively. Seeds were germinated by scarifying the water-impermeable seed coat. We obtained 100% germination of collected seeds. Although high seed infestation rate by a bruchid seed beetle, *Althaeus hibisci,* has been reported in the study area (Kudoh and Whigham, 1998), nonviable seeds rapidly lose their buoyancy (R. Shimamura, Tokyo Metropolitan University, unpublished data). Seedlings were grown in a greenhouse until they had 3–4 true leaves. Leaves were then harvested from the seedlings for allozyme analyses.

Allozyme and statistical analyses—Leaves were placed in a cooler box in the green house and then stored in the laboratory at 4°C prior to electrophoresis. Samples were analyzed within 2 d of collection. We analyzed all samples for three enzymes (esterase [EST], phosphoglucoisomerase [PGI], and phosphoglucomutase [PGM]) that showed consistently clear and genetically interpretable banding patterns in the 1997 study. Approximately 80 mg of leaf tissue was homogenized in 1.2 mL of a modified Shiraishi (1988) extraction buffer (0.1 mol/L tris-HCl [pH 7.5], 20% [v/v] glycerol, 1% [v/v] between 80, 10 mmol/L dithiothreitol [DTT], 0.1% [v/v] β-mercaptoethanol, and 75 mg/mL polyvinylpolypyrrolidone [PVPP]). The extracts were loaded on polyacrylamide vertical slab gels (Davis, 1964; Ornstein, 1964) after refining by centrifugation (15 000 rpm for 45 min at 1°C). Electrophoresis was carried out at 4°C, 11 mA/cm^2 for 180 min. Enzymes were stained following the protocols of Shiraishi (1988). The three putative loci (*Est*, *Pgi*, and *Pgm*) were scored for all individuals (see Kudoh and Whigham, 1997). *Est* and *Pgm* showed monomeric banding patterns with three and two alleles, respectively. *Pgi* showed a dimeric variation pattern with three alleles. Allelic variation at these loci was coded alphabetically with the most slowly migrating allozyme designated *a*.

We used Weir and Cockerham's (1984) estimates of Wright's (1951) *F* statistics (*F*, θ, and *f* for estimates of F_{IT}, F_{ST}, and F_{IS}, respectively) to characterize genetic subdivision among the three floating seed populations (sites A, B, and C). The overall extent of inbreeding in floating-seed populations (*F*) was partitioned into inbreeding due to nonrandom mating in each site (*f*) and inbreeding due to the correlation among alleles caused by their occurrence in the same site (θ). Calculations were made using FSTAT (Goudet, 1995). The significance of *F*, θ, and *f* per locus and overall loci were tested (Goudet, 1995) against the distribution of the null hypothesis, namely *F* (or θ, *f*) not > 0, obtained by permutations (Manly, 1991; Excoffier, Smouse, and Quattro, 1992; Hudson, Boss, and Kaplan, 1992). Using data from ten *H. moscheutos*

TABLE 1. Genotype and allele frequencies of seed samples collected from the different portions of Muddy Creek (A, upper stream; B, middle stream; C, lower stream). N = number of seeds.

Locus	Genotype/allele	Genotype/allele frequency Site A (N = 42)	Site B (N = 110)	Site C (N = 100)
Genotype frequency				
Pgi	*aa*	0.07	0.09	0.09
	ab	0.00	0.13	0.11
	ac	0.26	0.20	0.23
	bb	0.05	0.07	0.07
	bc	0.10	0.18	0.17
	cc	0.52	0.33	0.33
Pgm	*aa*	0.81	0.75	0.81
	ab	0.17	0.21	0.17
	bb	0.02	0.05	0.02
Est	*aa*	0.17	0.18	0.15
	ab	0.29	0.30	0.38
	ac	0.00	0.00	0.00
	bb	0.50	0.50	0.46
	bc	0.05	0.02	0.00
	cc	0.00	0.00	0.01
Allele frequency				
Pgi	*a*	0.20	0.26	0.26
	b	0.10	0.22	0.21
	c	0.70	0.52	0.53
Pgm	*a*	0.89	0.85	0.89
	b	0.11	0.15	0.11
Est	*a*	0.31	0.33	0.34
	b	0.67	0.66	0.65
	c	0.02	0.01	0.01

populations (Fig. 1) reported in Kudoh and Whigham (1997), we estimated gene flow between each population of established plants and the three floating-seed populations ($\hat{M}$) from a matrix of θ (Slatkin and Maddison, 1990; Slatkin, 1993) using FSTAT (Goudet, 1995). Standard genetic distances (Nei, 1972) between all pairs of the ten established populations and the three floating seed populations were calculated using POPGENE 1.31 (Yeh, Yang, and Boyle, 1999). A dendrogram was generated based on the distance matrix using the neighbor-joining method (Saitou and Nei, 1987) using PHYLIP 3.5c (Felsenstein, 1993). If the population is small and isolated, there is a higher probability of the loss of rare alleles by random genetic drift (Slatkin, 1985). The distribution of a rare allele, *Est-c*, across the ten established populations and the three floating-seed populations was recorded.

RESULTS

The three putative loci, *Est*, *Pgi*, and *Pgm*, were polymorphic in seeds sampled at each of the three sites, and allele frequencies were similar across seeds at the three sampling sites (Table 1). Significantly positive F for each locus and all loci combined indicated an overall excess of homozygotes in the floating-seed populations (Table 2). Significantly positive f was detected for each locus and for all loci combined (Table 2). No θ were significantly different from zero except for *Pgi*, for which a small deviation of θ from zero was detected (Table 2).

Gene flow ($\hat{M}$) calculated from the ten established populations to the three current-year floating-seed populations were highly variable depending on the seed-source population (Fig. 2a–c). High $\hat{M}$ values (>10) were detected from sites 1, 3, 4, 5, 9, and 10 for floating seeds at all three sampling sites (Fig. 2a–c). Sites 6, 7, and 8 showed relatively low $\hat{M}$ values for floating seeds at all three sites, and Site 2 showed a high $\hat{M}$ only for floating seeds at sampling site A (Fig. 2a–c). Figure 2D represents historical gene flow among established sites and

TABLE 2. Weir and Cockerham (1984) estimates of Wright's (1951) F statistics ($F = F_{IT}$, $\theta = F_{ST}$, $f = F_{IS}$) calculated for three seed samples taken from Muddy Creek.

Enzyme locus	F	θ	f
Pgi	0.189**	0.012*	0.179**
Pgm	0.149*	0.000	0.149*
Est	0.247**	−0.007	0.253**
All loci	0.203**	0.003	0.201**

*, ** Significant deviation of F statistics from zero (*$P < 0.05$, **$P < 0.001$).

was relatively high for sites 3, 4, 5, and 10 (Fig. 2d), all located near the tidal stream of the Muddy Creek (Fig. 1). A dendrogram generated by the neighbor-joining method demonstrated genetic similarity among the floating-seed populations and the established populations adjacent to Muddy Creek (Fig. 3). A rare allele, *Est-c*, was found in all floating-seed populations and was present in established populations at sites 3, 4, 9, and 10 (Table 1, Fig. 3).

DISCUSSION

Genetic structure of floating-seed populations—Overall, we detected significant positive deviation of two measures of inbreeding (F and f) from zero, but the measure of inbreeding among alleles caused by their occurrence in the same site (θ) was not significantly different from zero (Table 2). The overall excess of homozygotes, therefore, is mostly attributable to the excess of homozygotes within each site. The distances separating the three sampling sites for floating-seed populations ranged from ~300 m between sites A and B to 1200 m between sites B and C. The weak genetic structuring among floating-seed populations suggests long-distance (>1200 m) dispersal of seeds along Muddy Creek. Other investigators have found evidence that hydrochory results in effective long-distance seed dispersal at scales of hundreds of metres or kilometres (Waser, Vickery, and Price, 1982; Schneider and Sharitz, 1988; Nilsson, Gardfjell, and Grelsson, 1991; Lonsdale, 1993; Edwards, Wyatt, and Sharitz, 1994). The significant positive f (0.201) for all loci (Table 2) is consistent with the findings of Snow et al. (1996) who estimated a selfing rate for *H. moscheutos* at sites 1 and 2 (Fig. 1) of 36% based on an analysis of variation in seeds from 38 mature plants. Spatial separation of anthers and stigmas prevents autogamous pollination in *H. moscheutos* flowers, but pollinators transfer pollen within and among flowers of the same plant (geitonogamous pollination), and seeds can be sired by inbreeding (Spira, 1989). We therefore conclude that the positive f detected in the floating-seed populations is attributable to the breeding system of the species. On the other hand, a positive f for floating-seed populations contrasts with an f of almost zero (0.017) for populations of established flowering plants (Kudoh and Whigham, 1997). The difference in f values between the floating-seed populations (this study) and the mature plant populations (Kudoh and Whigham, 1997) may be explained by differential survival of seedlings that are produced by inbreeding and by outcrossing. Inbred progeny of *H. moscheutos* were reported to have lower growth rates than outcrossed progeny (Snow and Spira, 1993).

Current year seed dispersal and historical gene flow—In our previous study (Kudoh and Whigham, 1997), we estimated

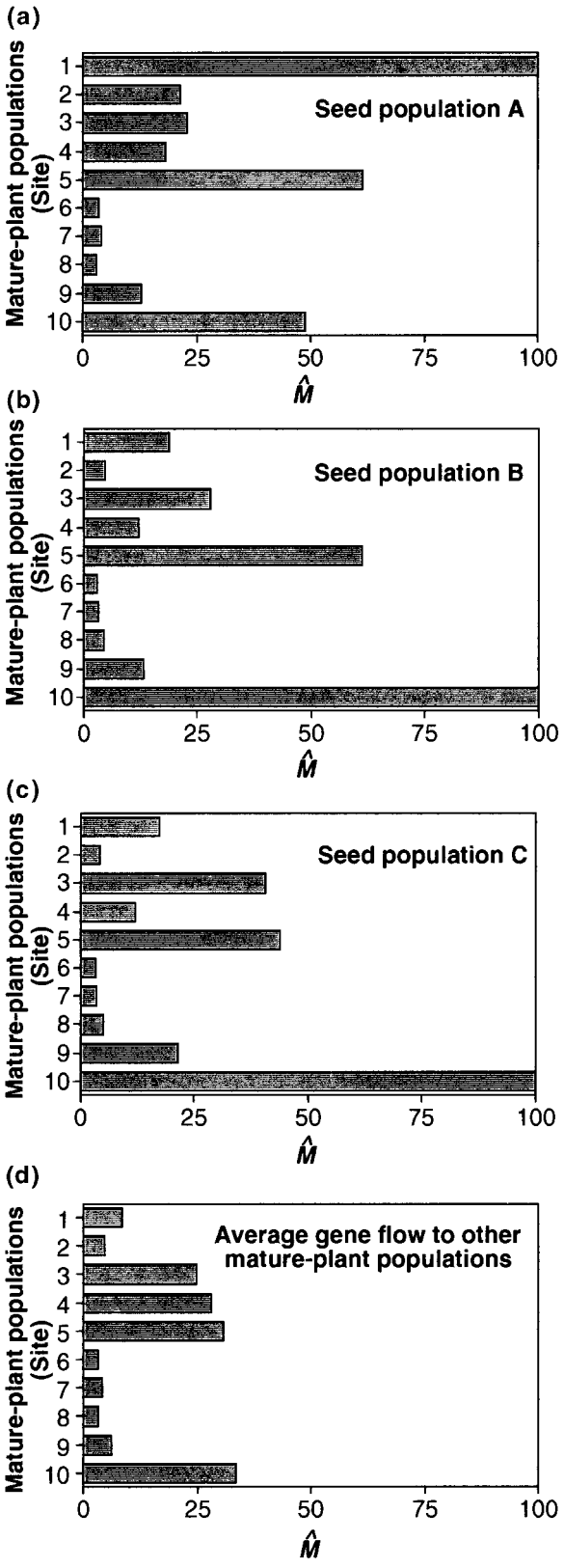

Fig. 2. Gene flow ($\hat{M}$) calculated between ten mature-plant populations and three floating-seed populations (a–c), and gene flow ($\hat{M}$) among ten mature-plant populations (d). Graphs a–c show contributions of ten mature-plant populations to the current-year floating-seed populations, whereas graph d shows historical gene flow among the ten mature-plant populations. In d, average $\hat{M}$ values from each population to the other nine sites are shown.

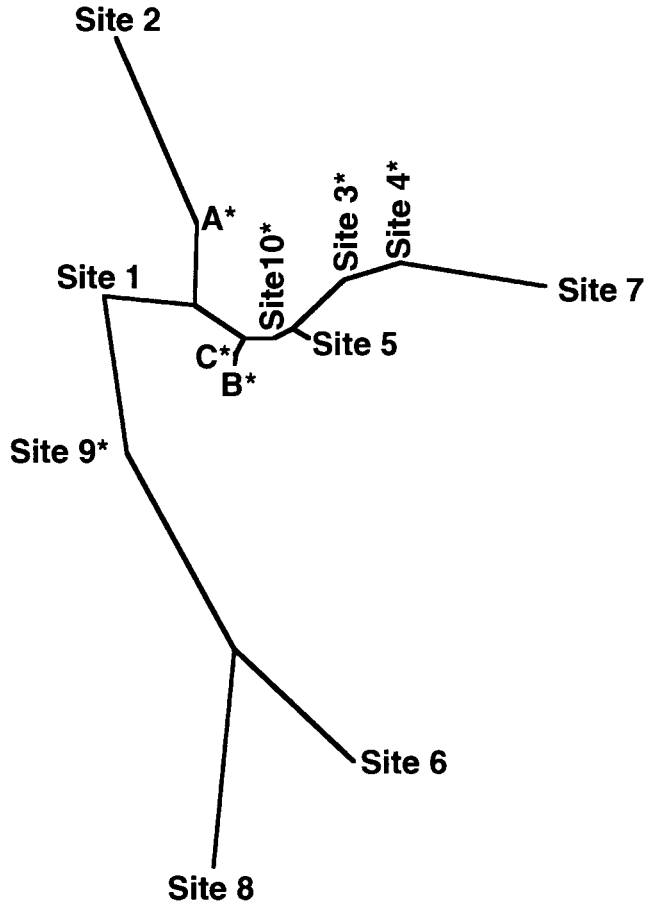

Fig. 3. Dendrogram generated by the neighbor-joining method (Saitou and Nei, 1987). The distance matrix was calculated by Nei's standard distance (Nei, 1972). Mature-plant populations or floating-seed populations with similar genetic composition are shown as neighbors on the dendrogram. Asterisks indicate populations where a rare allele, *Est-c*, was found.

gene flow averaged over long periods of time using allozyme variation among established populations. We do not know the age of the established populations but they have been present for at least 22 yr (D. Whigham, personal observation). Estimated historical gene flow (Kudoh and Whigham, 1997) was greatest between populations that were adjacent to the tidal stream (see Fig. 2d). Populations that were not close to the tidal stream appeared to be relatively isolated and had reduced gene flow with other sites (Fig. 2d). Contributions to the current-year floating seeds from populations adjacent to the tidal stream (sites 3, 4, 5, and 10) were also detected in this study. Thus, the current-year seed dispersal (Fig. 2a–c) and historical gene flow (Fig. 2d) showed a similar pattern (Fig. 2). These results indicate that populations along the tidal stream may function as a single genetic metapopulation by extensive and long-distance seed exchanges between them (Fig. 3). The relatively large effective size of the metapopulation may act as a reserve of genetic variation in the system by reducing the effects of random genetic drift. The distribution of a rare allele, *Est-c*, also supports this conclusion (Fig. 3). Although site 6 is adjacent to tidal stream, it exhibits a relatively high degree of genetic isolation and contributes weakly to the floating-seed population. This pattern may be explained by a stochastic reduction in population size and by a lower seed output in the

site. The site is located most downward along the tidal stream among the populations that have no freshwater input to the site (Fig. 1) and is characterized by low plant density and small plant size (Kudoh and Whigham, 1997).

The contributions of sites 1 and 2 to floating-seed populations in the upstream portion of Muddy Creek (Fig. 2a and b) and contributions of site 9 to the lower portion of Muddy Creek (Fig. 2c) were larger than detected in the historical gene flow (Fig. 2d). These differences may represent unidirectional movement of seeds from sites 1, 2, and 9 to the floating-seed populations in the tidal stream. Sites 1 and 2 are hydrologicaly linked to Muddy Creek by freshwater flow and unidirectional seed dispersal would be expected to occur in the autumn when seeds are dispersed and when freshwater flows are common, especially during high volume rain events. Although site 9 is periodically inundated, seed movement into and out of the site may be asymmetrically reduced by wetland vegetation and thick litter (Jordan, Whigham, and Correll, 1989). Site 9 is currently more hydrologically linked to Muddy Creek than it was at the time of our earlier study (Kudoh and Whigham, 1997). A high level of muskrat activity in the wetlands near site 9 has resulted in the almost complete loss of *Typha angustifolia*, the dominant emergent species, and establishment of a direct hydrologic link between site 9 and Muddy Creek. Periodic long-distance connections between isolated populations and tidal creeks have, however, also been demonstrated. Huiskes et al. (1995) studied seed movements in tidal salt marshes using floating nets. They found that few propagules of wetland plants were transported into the marsh with the incoming tide and a significantly higher number of propagules were transported out of the marsh with the ebb currents. M. Leck (Rider University, personal communication), working in a created tidal freshwater wetland in New Jersey, found that large numbers of floating seeds entered during flood tides.

In conclusion, the importance of hydrochory in this system was suggested both in current-year seed dispersal and historical gene flow. Seed exchange was estimated to be greatest between populations that are adjacent to the tidal stream. Populations that were not close to the tidal stream are relatively isolated with reduced historical gene flow, but they can contribute periodically to the floating-seed populations in the tidal stream, at least for the year of our study. Although we successfully compared historical gene flow and current-year seed dispersal among multiple populations, this study suggests that yearly variation in seed dispersal patterns among multiple populations needs to be evaluated in the further studies.

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