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Evolution

Pollen-mediated introgression and hybrid speciation in Louisiana irises

(introgressive hybridization/*Iris*/polymerase chain reaction)

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ABSTRACT **Populations of the "Louisiana iris" species *Iris fulva*, *I. hexagona*, and *I. nelsonii* were examined genetically to test for interspecific gene flow between *I. fulva* and *I. hexagona*, for pollen- versus seed-mediated introgression between these species, and for the presumed hybrid origin of *I. nelsonii*. Genetic markers were identified by using both a polymerase chain reaction-like method that allows the identification of random, nuclear markers and standard polymerase chain reaction experiments involving specific chloroplast DNA (cpDNA) oligonucleotides. Restriction endonuclease digestions of the cpDNA amplification products resolved diagnostic restriction site differences for *I. fulva* and *I. hexagona*. The distribution of the species-specific nuclear markers supports a hypothesis of bidirectional introgression between *I. fulva* and *I. hexagona*. Thus, individuals analyzed from a contemporary hybrid population demonstrate multilocus genotypes that are indicative of advanced-generation hybrid individuals. Furthermore, several markers from the alternate species were present in low frequency in one allopatric population each of *I. fulva* and *I. hexagona*. Data from the nuclear analysis also support the hypothesized hybrid origin of *I. nelsonii* from the interaction of *I. fulva* and *I. hexagona*. Finally, cpDNA data support the hypothesis that the localized and the dispersed introgression are largely due to pollen transfer. In addition to the biological implications, this study demonstrates the power of the polymerase chain reaction methodology for the rapid identification of random and specific genetic markers for testing evolutionary genetic hypotheses.**

Natural hybridization has alternatively been viewed as adaptive or maladaptive in various species complexes (1–7), and a limited number of studies have utilized direct experimental analysis to define the relative "fitness" of parental and hybrid classes (8–10). Additionally, the occurrence of introgressive hybridization (i.e., introgression; ref. 11) has led to inferences concerning adaptive consequences of natural hybridization (1, 12). These inferences are based upon the hypothesis that introgression is both an outcome of natural hybridization and also an intermediate step for other evolutionary events. Thus, introgression may be a causative factor in a reduction of barriers to sexual reproduction (13), a change in the fitness of the taxon that is introgressed (1, 8), a range expansion of the introgressed form into habitats that differ from those of the parental taxa (1), or a hybrid speciation event (3, 14).

In the present analysis we have examined the genetic consequences of interspecific hybridization between the Louisiana irises *Iris fulva* and *I. hexagona*. Natural hybridization between these species takes place in southern Louisiana, where these two species co-occur (15–21). Contact between *I. fulva* and *I. hexagona* is due to the interdigitation of bayous originating from the Mississippi River (*I. fulva* habitat) and the intercoastal, freshwater swamp and marsh environments (*I. hexagona* habitats). Although the interaction between these two species was originally used to exemplify the process of introgressive hybridization (16, 17), later morphological and cytological studies did not detect the transfer of genetic material between these two species (20). However, recent molecular studies (19, 21) have supported the conclusions of Anderson (17) with regard to the occurrence of both localized and dispersed introgression.

The detection of introgression, in particular with reference to the Louisiana iris species, relies upon the identification of diagnostic markers for the taxa under analysis. The potential importance of molecular markers for such studies has been emphasized by numerous authors (22, 23). We used a combination of the polymerase chain reaction (PCR) method and restriction fragment length analysis to define nuclear DNA (nDNA) and chloroplast DNA (cpDNA) markers for Louisiana iris species. Data from these analyses allowed a test for (*i*) interspecific gene flow and (*ii*) hybrid speciation involving the Louisiana iris species *I. fulva* and *I. hexagona*, and a putative hybrid species, *I. nelsonii*. Further, the cpDNA analysis facilitated a test for seed- versus pollen-mediated gene flow between *I. fulva* and *I. hexagona*.

MATERIALS AND METHODS

Population Samples and DNA Isolation. The population localities (all in Louisiana) are as follows (n is the sample size for each population surveyed): *I. fulva*/1, East Baton Rouge Parish, Bayou Paul Road, $n = 10$; *I. fulva*/2, Terrebonne Parish, 7.6 km west of junction of Highway (Hwy) 20 and Hwy 311 on Hwy 311, $n = 10$; *I. fulva*/3, Lafourche Parish, 6.8 km south of Kraemer on Hwy 307, $n = 12$; *I. hexagona*/1; St. Mary Parish, junction of Cypress Pass Bayou and Wax Lake Bayou, $n = 5$; *I. hexagona*/2, St. Mary Parish, junction of Wax Lake Bayou and Big Hog Bayou, $n = 5$; *I. hexagona*/3, St. Mary Parish, 0.3 km west of the mouth of Big Hog Bayou and ≈100 m from Atchafalaya Bay, $n = 10$; *I. hexagona*/4; St. Martin Parish, 11 km north of Morgan City on Hwy 70, $n = 6$; *I. hexagona*/5, Terrebonne Parish, 11 km south of Houma, $n = 14$; *I. nelsonii*, Vermilion Parish, 0.3 km from junction of Hwy 330 and P-2-7 on Hwy 330, $n = 8$; hybrid population, Assumption Parish, 1 km south of junction of Hwy 662 and Hwy 398 on Hwy 662, $n = 42$. DNA isolation was carried out using leaf material and the procedure of Arnold *et al.* (19). This resulted in the collection of both nDNA and cpDNA.

DNA Amplification, Restriction Endonuclease Analysis, Gel Electrophoresis, and Detection of Amplification Products. *nDNA analysis.* The amplification of the random, nuclear

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Abbreviations: nDNA, nuclear DNA; cpDNA, chloroplast DNA; PCR, polymerase chain reaction; RAPD, random amplified polymorphic DNA.

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markers [i.e., random amplified polymorphic DNA (RAPD) analysis; ref. 24] was accomplished by the methods of Williams *et al.* (24). This protocol is "PCR-like" in that it involves the use of a single, rather than a pair, of oligonucleotides to amplify regions of the genome. The oligonucleotide primers are arbitrary in that they are chosen without any consideration of the relative nucleotide sequence of the genome being assayed (24).

Reaction mixtures (25 μl) contained 10 mM Tris·HCl (pH 8.3); 50 mM KCl; 1.9 mM $MgCl_2$; 0.001% gelatin; 0.1 mM each dATP, dTTP, dCTP, and dGTP; 200 μmol of a single, unique primer (MA-1, 5′-TGCTCACTGA-3′; MA-2, 5′-TGGACACTGA-3′; or MA-3, 5′-ACCTCCAGCACTGTCA-3′); 25 ng of genomic DNA; and 0.42–1.25 units of *Thermus aquaticus* (*Taq*) DNA polymerase (Perkin–Elmer/Cetus). The mixtures were incubated in a Perkin–Elmer/Cetus DNA thermal cycler programmed for 45 cycles of 1 min at 92°C, 1 min at 35°C (MA-1 and MA-2) or 1 min at 55°C (MA-3), and 2 min at 72°C. The amplification products were run in a 1.4% agarose gel, in the presence of ethidium bromide, at 25–35 V for ≈16 hr. The gels were then photographed using a UV light source and each individual was scored for the presence or absence of the marker products.

Several experimental protocols were used to determine whether or not the amplification products originated from *Iris* genomic elements or were artifacts of the amplification procedure. (*i*) Control samples that had each of the reaction constituents, except genomic DNA, were run along with the samples containing DNA. These samples never contained the amplification products that were present in the samples containing the genomic DNA. (*ii*) Experiments were run to test the effect of Mg^{2+}, DNA, and *Taq* polymerase concentration on the amplification experiments. The DNA and Mg^{2+} affected the size and number of amplification products (M.L.A., unpublished data); varying concentrations of *Taq* polymerase demonstrated no such effect. Thus, samples containing Mg^{2+} at 1.5–4.7 mM demonstrated a wide array of products. Some differences were observed in the amplification products produced in samples containing 2.5 ng to 1 μg of DNA. In spite of this variation, consistent, reproducible results were obtained by standardizing the reaction conditions to those listed above. These results were consistent between amplification experiments where the same samples were reexamined. The final test for the reproducibility and genetic characteristics of the various amplification products involved the examination of experimental F_1 individuals. For the primers and the experimental methods reported above, each of the F_1 individuals demonstrated a combination of the diagnostic *I. fulva* and *I. hexagona* markers.

cpDNA analysis. The identification of the cpDNA markers characteristic for *I. fulva* and *I. hexagona* involved the use of oligonucleotide primers homologous to the region containing the ribulose-1,5-bisphosphate carboxylase gene (i.e., *rbcl*; Z1, 5′-ATGTCACCACAAACAGAAACTAAAGCAAGT-3′, ref. 25; ORF106, 5′-ACTACAGATCTCATACTACCCC-3′, ref. 26). The Z1 primer is identical to positions 1–30 of the *Zea mays rbcl* gene (25). The ORF106 primer is identical to the *Oryza sativa* conserved open reading frame ORF106 (26). The expected size for the amplification product with these two primers was ≈2–3 kilobases (kb), depending on the length of intervening, noncoding sequences (M. Clegg and M. Durbin, personal communication). PCR amplification of this cpDNA region was accomplished as follows. Reaction mixtures (100 μl) contained 10 mM Tris·HCl (pH 8.3); 50 mM KCl; 1.5 mM $MgCl_2$; 0.001% gelatin; 0.1 mM each dATP, dTTP, dCTP, and dGTP; 50 pmol of each primer; 1–2 μg of genomic DNA; and 2.5 units of *Taq* DNA polymerase (Perkin–Elmer/Cetus). As with the RAPD experiments, amplification was carried out using a Perkin–Elmer/Cetus DNA thermal cycler. The thermal cycler was programmed for 1 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 60°C, and 4 min at 72°C, with a final 7 min at 72°C. The *I. fulva* and *I. hexagona* cpDNA amplification products were used in restriction endonuclease digests to attempt to identify diagnostic restriction site or length differences. Initial digests involved 20 enzymes (*Ase* I, *Bcl* I, *Hpa* II, *Hph* I, *Alu* I, *Bam*HI, *Bst*UI, *Eco*RI, *Hae* III, *Hha* I, *Hin*dIII, *Hin*fI, *Hin*PI, *Mbo* I, *Nde* I, *Pst* I, *Rsa* I, *Sac* I, *Sau*3A1, and *Taq* I; New England Biolabs).

RESULTS

Identification of Diagnostic RAPD Markers for *I. fulva* and *I. hexagona*. To identify diagnostic markers for *I. fulva* and *I. hexagona*, seven unique, 10- to 16-base-pair (bp) primers were used to amplify genomic DNA from these two species. Five of the seven primers were successful in identifying differences between these two species (Fig. 1). Three primers (MA-1, MA-2, and MA-3) that demonstrated species-specific amplification products were chosen to survey experimental F_1 hybrids and naturally occurring parental and hybrid populations. The pattern of inheritance for the MA-1 and MA-2 primers was tested using the experimental F_1 hybrids ($n = 6$) recovered from crosses of *I. fulva* and *I. hexagona*. Each of the fragments that were diagnostic for the two parental taxa was present in the hybrids (Fig. 2). Experiments using the MA-3 primer to analyze the F_1 hybrid individuals also revealed the expected patterns of inheritance (M.L.A., unpublished data).

The consistency and reproducibility of the species-specific amplification products were tested in multiple (five or more) experiments with several *I. fulva* and *I. hexagona* individuals, for each of the primers; each of these experiments

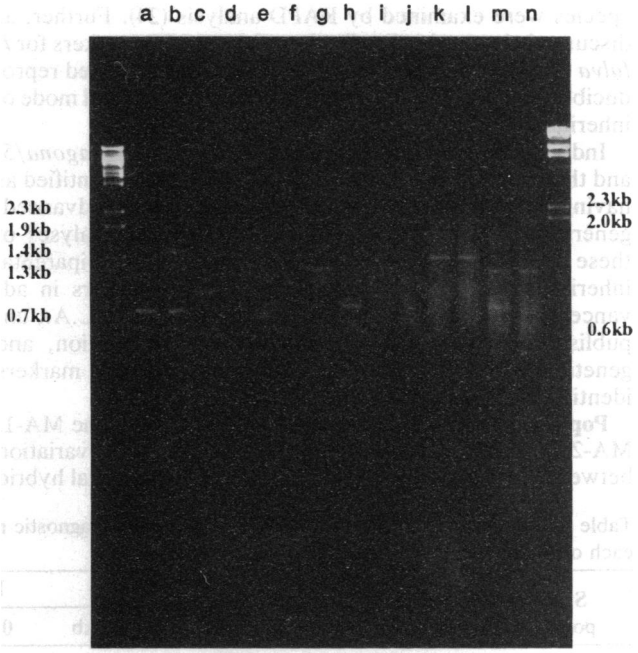

FIG. 1. Results from random primer amplifications using seven unique primers. Lanes a, c, e, g, i, k, and m and lanes b, d, f, h, j, l, and n show reaction products from amplifications using *I. fulva* and *I. hexagona* genomic DNA, respectively. A single, unique primer was used to produce the amplification products in each pair of lanes. Differences in the *I. fulva* and *I. hexagona* amplification products are seen in lanes a and b, e and f, g and h, i and j, and m and n. The primers chosen for further analysis were MA-1 (lanes e and f), MA-2 (lanes g and h), and MA-3 (data not shown). Lanes at extreme left and right contain λ phage DNA digested with *Bst*EII (left) or *Hin*dIII (right) used as molecular size markers.

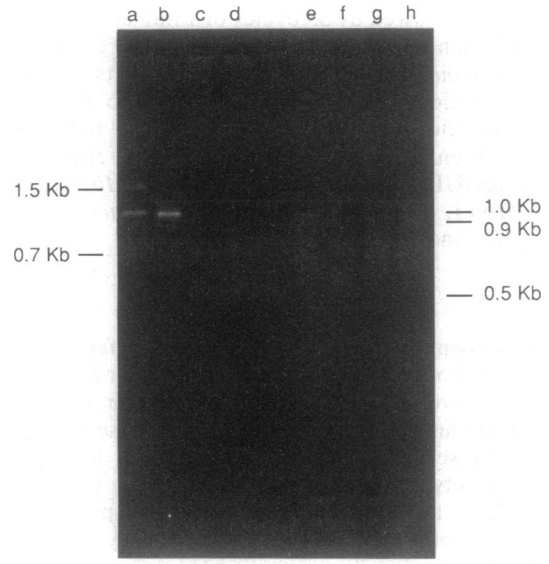

FIG. 2. Amplification products of DNA from *I. fulva* (lanes a and e), *I. hexagona* (lanes b and f), and two F_1 individuals (lanes c and d and lanes g and h) with the random primers MA-1 (lanes a–d) and MA-2 (lanes e–h). Note the presence of the *I. fulva* MA-1 markers (1.5 and 0.7 kb) in the F_1 individuals, and the presence of both the *I. fulva* MA-2 (1.0 and 0.9 kb) and *I. hexagona* MA-2 (0.5 kb) diagnostic products in the two F_1 individuals.

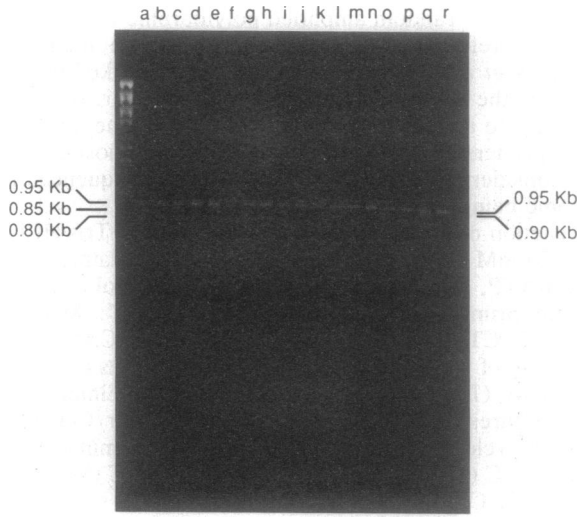

FIG. 3. Amplification products from experiments using the MA-3 primer and genomic DNA from individuals of population *I. fulva*/1 (lanes a–i) and individuals of populations *I. hexagona*/1 and 2 (lanes j–r). Eight of the *I. fulva* individuals (lanes a–g and i) have the 0.95-, 0.85-, and 0.80-kb amplification products. The remaining *I. fulva* individual (lane h) possesses only the 0.95- and 0.80-kb products. Eight of the *I. hexagona* individuals (lanes j–p and r) have only the 0.95-kb product, with the remaining individual (lane q) demonstrating both the 0.95- and 0.90-kb products. Lane at left contains size markers.

resolved the same products. The reproducibility of these results could also be seen for the *I. fulva* and *I. hexagona* individuals used as standards in each experiment. These individuals always demonstrated the same pattern of amplification products throughout the study. These results are consistent with those of a previous study in which several species were examined by RAPD analysis (24). Further, as discussed above, the distribution of the RAPD markers for *I. fulva* and *I. hexagona* in the F_1 hybrids demonstrated reproducible results that were consistent with a biparental mode of inheritance.

Individuals from populations *I. fulva*/3, *I. hexagona*/5, and the hybrid population have previously been identified as having multilocus genotypes characteristic of advanced-generation hybrids (21). Similarly, the RAPD analyses of these populations revealed patterns explicable by biparental inheritance and segregation of the RAPD markers in advanced-generation hybrid individuals (Table 1; M.L.A., unpublished data). Biparental inheritance, segregation, and genetic linkage have also been reported for RAPD markers identified for the soybean genome (24).

Population Analysis Using the RAPD Markers. The MA-1, MA-2, and MA-3 primers were also used to assess variation between *I. fulva*, *I. hexagona*, *I. nelsonii*, and natural hybrid individuals. For example, Fig. 3 depicts the variation in an *I. fulva* and an *I. hexagona* population for amplification products derived using the MA-3 primer. A total of 122 individuals were screened for each of the three primers. These samples represented individuals from populations of *I. fulva*, *I. hexagona*, *I. nelsonii* (a putative hybrid species), and a region of overlap between *I. fulva* and *I. hexagona*. Table 1 contains frequency data for the MA-1, MA-2, and MA-3 amplification products for each of the populations surveyed. Each of the *I. fulva* individuals surveyed possessed the MA-1, 1.5-kb fragment, the MA-2, 1.0-kb and 0.9-kb fragments, and the MA-3, 0.8-kb fragment. Further, all but one of these individuals demonstrated the MA-1, 0.7-kb amplification product. The *I. fulva* individuals from populations 1 and 2 lacked the MA-2, 0.5-kb fragment; however, there was a significant frequency of this marker in *I. fulva* population 3. In contrast, all the individuals examined from the *I. hexagona* populations 1–4 lacked the MA-1, 1.5-kb and 0.7-kb markers, the MA-2, 1.0-kb and 0.9-kb markers, and the MA-3, 0.8-kb marker but possessed the MA-2, 0.5-kb fragment. The remaining *I. hexagona* population (*I. hexagona*/5) demonstrated significant frequencies of the MA-1 markers as well as the MA-2, 1.0-kb and 0.9-kb amplification products. The present-day

Table 1. Frequencies of the *I. fulva* and *I. hexagona* diagnostic markers produced using the random primers MA-1, MA-2, and MA-3 for each of the populations surveyed

Species/ population	MA-1		MA-2			MA-3			
	1.50 kb	0.70 kb	1.00 kb	0.90 kb	0.50 kb	0.95 kb	0.90 kb	0.85 kb	0.80 kb
I. fulva/1	1.00	0.90	1.00	1.00	0	1.00	0	0.90	1.00
I. fulva/2	1.00	1.00	1.00	1.00	0	1.00	0	0.40	1.00
I. fulva/3	1.00	1.00	1.00	1.00	0.33	1.00	0	0	1.00
I. hexagona/1	0	0	0	0	1.00	1.00	0.20	0	0
I. hexagona/2	0	0	0	0	1.00	1.00	0.20	0	0
I. hexagona/3	0	0	0	0	1.00	1.00	0	0	0
I. hexagona/4	0	0	0	0	1.00	0.80	0.80	0	0
I. hexagona/5	0.08	0.50	0.21	0.21	1.00	1.00	0.50	0	0
I. nelsonii	0.60	0.60	1.00	1.00	0.14	1.00	0	0.13	0.13
Hybrid population	0.33	0.17	0.22	0.22	0.93	1.00	0.12	0	0.24

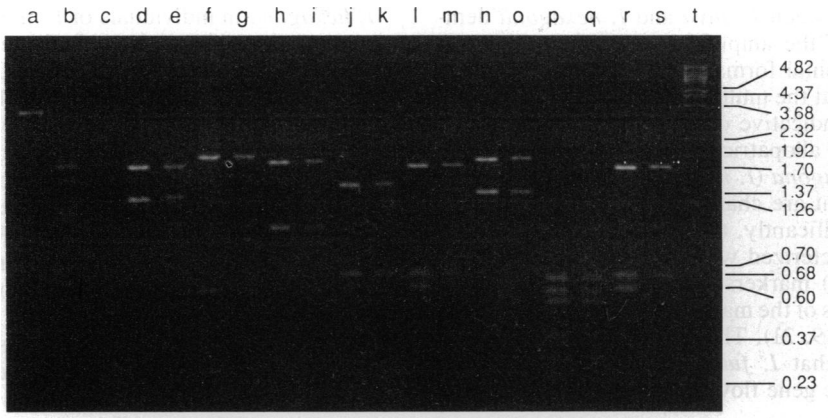

FIG. 4. Results of restriction endonuclease digestions of cpDNA amplification products from *I. fulva* (lanes b, d, f, h, j, l, n, p, and r) and *I. hexagona* (lanes c, e, g, i, k, m, o, q, and s). Lanes a and t contain undigested cpDNA amplification product from *I. fulva* and λ phage DNA digested with *Bst*EII, respectively. The restriction enzymes used were *Alu* I (lanes b and c), *Bam*HI (lanes d and e), *Bst*UI (lanes f and g), *Eco*RI (lanes h and i), *Hae* III (lanes j and k), *Hha* I (lanes l and m), *Hin*dIII (lanes n and o), *Hin*fI (lanes p and q), and *Hin*PI (lanes r and s). Numbers at right indicate sizes in kilobases.

hybrid population has relatively intermediate frequencies of the markers, although biased toward the values present in *I. hexagona* populations 1–4. Finally, the *I. nelsonii* population sample has intermediate frequencies for both of the MA-1 markers, the MA-2, 0.5-kb marker and the MA-3, 0.8-kb marker but is fixed for the MA-2, 1.0-kb and 0.9-kb products.

Identification of Diagnostic cpDNA Haplotypes for *I. fulva* and *I. hexagona*. The amplification products from the *rbcl* regions of *I. fulva* and *I. hexagona* were both ≈3500 bp long (Fig. 4; M.L.A., unpublished data). Further, for 18 of 20 restriction endonucleases the digestion patterns for the two species were identical (Fig. 4; M.L.A., unpublished data). The enzymes chosen for the survey of cpDNA variation were *Hha* I and *Hin*PI. These endonucleases have identical recognition sites and demonstrate identical restriction patterns when used in digests of either *I. fulva* or *I. hexagona* amplification products (Fig. 4). The difference in the *Hha* I/*Hin*PI restriction digest patterns for the two iris species appears to be due to a restriction-site loss or gain. Therefore, the *I. fulva* and *I. hexagona* haplotypes share bands of approximately 1700, 680, and 230 bp (Fig. 4). In contrast, *I. fulva* possesses an ≈600-bp restriction fragment, while *I. hexagona* has an ≈370-bp band (Fig. 4). Since the *I. fulva* and *I. hexagona* amplification products are the same size (Fig. 4; M.L.A. unpublished data), the banding difference in the two haplotypes is most likely due to the presence of an additional restriction site in the *I. hexagona* product that results in the cleaving of the 600-bp band seen in *I. fulva* into a 370-bp and a 230-bp fragment. This additional 230-bp fragment would comigrate with the 230-bp fragment present in both species. The mode of genetic transmission of the cpDNA was tested by assaying cpDNA haplotypes of experimental F_1 individuals for which the maternal (*I. fulva*) and paternal (*I. hexagona*) parents were known (M.L.A., unpublished data). Each of these individuals had the *I. fulva* haplotype.

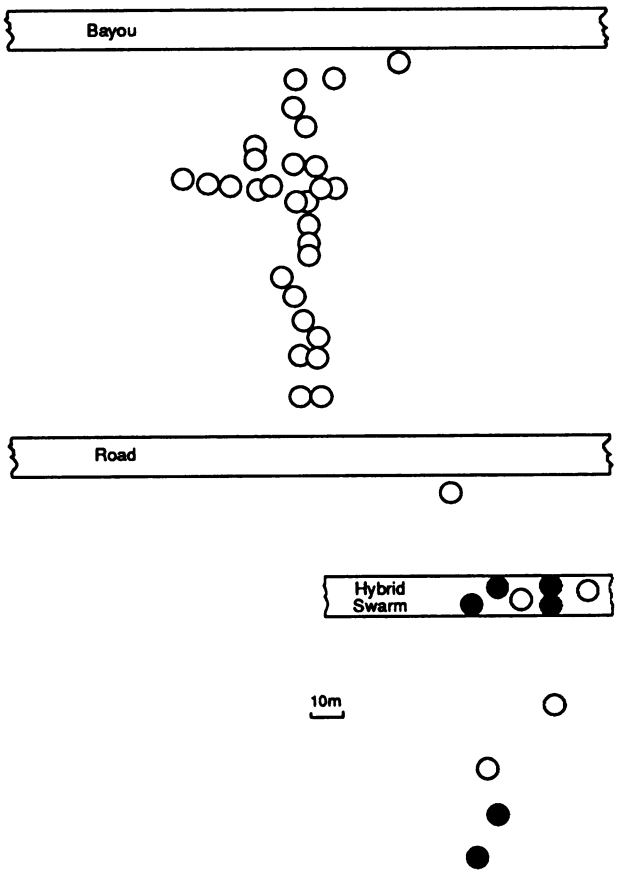

FIG. 5. Distribution and cpDNA haplotypes across a transect through the contemporary *I. fulva* × *I. hexagona* hybrid population. Each circle represents an individual plant. Open circles indicate the presence of the *I. hexagona* cpDNA haplotype (IH); filled circles reflect the presence of the *I. fulva* cpDNA haplotype (IF).

Distribution of cpDNA Haplotypes in Iris Populations. All but one of the populations surveyed for cpDNA variation showed a single haplotype. The *I. fulva* and *I. nelsonii* samples were characterized by the haplotype resolved for the *I. fulva* individuals (using *Hha* I and *Hin*PI) in Fig. 4 ("IF" haplotype). In contrast, the *I. hexagona* populations demonstrated only the haplotype seen for the *I. hexagona* individual in Fig. 4 ("IH" haplotype). The hybrid population had a mixture of the two haplotypes but contained a preponderance of the IH marker (Fig. 5).

DISCUSSION

Nuclear Variation and Introgressive Hybridization. The Louisiana iris species *I. fulva* and *I. hexagona* have been used as the paradigm for the process of introgressive hybridization (17). Furthermore, recent molecular and biochemical analyses have detected apparent interspecific gene flow in areas of parapatry and allopatry (19, 21). The distribution of the species-specific RAPD markers (Table 1) also supports the hypothesis that interspecific gene flow has occurred between *I. fulva* and *I. hexagona*. First, the population from

the area of overlap between *I. fulva* and *I. hexagona* demonstrates a mixture of the amplification products that are diagnostic for the parental forms. This is in accord with a previous conclusion that the multilocus genotypes present in this population were indicative of advanced generation hybrids (21). Second, one allopatric population each of *I. fulva* (*I. fulva*/3) and *I. hexagona* (*I. hexagona*/5) possesses amplification products that are characteristic for the alternate species (Table 1). Significantly, these two populations have previously been characterized with molecular (rDNA) and biochemical (allozyme) markers and have been shown to possess low frequencies of the markers that are diagnostic for the alternate species (19, 21). Thus, the present study supports the conclusion that *I. fulva* and *I. hexagona* have undergone interspecific gene flow resulting in localized and dispersed introgression.

The Distribution of cpDNA Variation and the Mediation of Introgressive Hybridization. Introgressive hybridization between plant species differs from animal systems with respect to the mechanisms available for the gene transfer. Thus, it is possible for gene flow to occur through the transfer of pollen or through the transport of seeds into populations of the alternate species, followed by pollen exchange (27). However, the relative contribution of pollen and seed movement to gene flow within and between plant species may vary widely due to the mode of transport of pollen and seeds (28, 29).

The cpDNA analysis of *I. fulva*, *I. hexagona*, and hybrid populations allowed a test for seed- versus pollen-mediated introgression. The cpDNA represents a diagnostic marker for the seed parent due to the cytoplasmic transmission of chloroplasts. Maternal inheritance has been detected in a large majority of, but not all, plant species (30). We tested for the mode of inheritance by analyzing cpDNA markers in a sample of F_1 hybrids ($n = 6$) derived from a cross where an *I. fulva* individual was the maternal parent and an *I. hexagona* individual was the pollen source. The hybrid individuals possessed only the IF cpDNA haplotype (M.L.A., unpublished data).

Each of the *I. fulva* populations, including *I. fulva*/3, had only the IF haplotype. In contrast, the *I. hexagona* populations 1–5 had only the IH haplotype. This finding supports the hypothesis that the nuclear introgression in *I. fulva*/3 and *I. hexagona*/5 (refs. 19 and 21; present analysis) has resulted from pollen- rather than seed-mediated gene flow. The only population that possessed a combination of these haplotypes was the contemporary hybrid population (Fig. 5), and within this population the two haplotypes showed a patchy distribution. For example, the portion of the hybrid population between the bayou channel and the road possesses a large proportion of hybrid, nuclear genotypes (refs. 19 and 21; present study), but only the IH cpDNA haplotype is present (Fig. 5). Therefore, the individuals sampled from this region are exclusively the products of pollen transfer from *I. fulva* or hybrid plants onto *I. hexagona* flowers. The other portion of this hybrid population possesses a mixture of the IF and IH haplotypes. This may indicate the involvement of both seed- and pollen-mediated gene flow into this area of the population or may reflect the historical contact point between the two species.

Distribution of the RAPD Markers and cpDNA Haplotypes: A Test for Hybrid Speciation. An examination of morphological and cytological characteristics for individuals of *I. nelsonii* led to the hypothesis that this species was a stabilized hybrid derivative of *I. fulva* and *I. hexagona* (31). A recent analysis of allozyme variation supported this hypothesis in that alternate allozyme markers from these two species were present in a population of *I. nelsonii* (21). Data from the present analysis also support the hypothesis of a hybrid origin for *I. nelsonii*. Thus, we detected the co-occurrence of alternative RAPD markers characteristic of either *I. fulva* or *I. hexagona* in individuals of *I. nelsonii*. The cpDNA haplotypes present in *I. nelsonii* indicated bias in the direction of the hybridization event(s) that produced this hybrid derivative. An examination of cpDNA haplotypes present in *I. nelsonii* detected only the IF haplotype. Thus, the origin of *I. nelsonii* appears to have involved *I. fulva* as the maternal (seed) parent and *I. hexagona* as the paternal (pollen) parent.

PCR Methodology and Evolutionary Genetic Analyses. In the present study, we have used PCR methodology to identify and analyze both random, nDNA markers and specific cpDNA restriction fragment length polymorphisms. Data from this study were used to test several evolutionary hypotheses concerning hybridization, introgression, hybrid speciation, and the process mediating interspecific gene flow. Significantly, the results from this analysis are an empirical demonstration of the potential power and applicability of PCR-based technology for studies of the evolutionary genetics of natural populations.

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