HOMOPLOID HYBRID SPECIATION
IN LOUISIANA IRIS

DISSERTATION

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by

Sunni J. Taylor, M.S.

San Marcos, TX
December 2012
HOMOPLOID HYBRID SPECIATION
IN LOUISIANA IRIS

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ACKNOWLEDGEMENTS

This work was made possible with the help of a number of people. Thank you to my committee for invaluable advice and comments. I am indebted to each person that volunteered to go to the swamp or repot plants with me. Yvonne and Reverend Roy Howard Martin wore out their mud boots setting up many of the projects detailed below as well as many more projects that failed or are still in progress. I also thank them profusely for producing and raising their son, Noland Martin, who would ultimately become such a faultless advisor. Thanks to Mom, Heathie, Margaret, and many other friends that spent many late nights repotting the thousands of irises involved in these projects. Undergraduate students were integral to the success of these projects and are named in the acknowledgments under the projects that they helped complete, including two undergraduates that are authors on the projects that they worked on.

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ABSTRACT

HOMOPLOID HYBRID SPECIATION

IN LOUISIANA IRIS

by

Sunni J. Taylor, M.S.

Texas State University-San Marcos

December 2012

SUPERVISING PROFESSOR: NOLAND MARTIN

As hybridization necessarily occurs in sympathy, hybrid species may represent the most frequent mechanism of sympatric speciation, and, as such, hybrid speciation events present the opportunity to study the evolution of reproductive isolation in sympathy. A number of purported cases of homoploid hybrid speciation in both plants and animals have recently been reported, suggesting that this process may be more common than once believed. The limited number of well-documented studies of reproductive isolation between a homoploid hybrid species and its progenitors yield varying patterns of reproductive isolation. In some taxa, strong fertility selection in early generation hybrids may yield a hybrid lineage that is isolated from its progenitors by strong postzygotic
isolation. Alternatively, ecological barriers may result in reproductive isolation between a hybrid lineage and its progenitors. Here, I examined homoploid hybrid speciation and reproductive isolation between a purported homoploid hybrid species, *Iris nelsonii*, and one of its progenitors, *Iris hexagona*. In order to investigate homoploid hybrid speciation in this group, I used population genetic surveys to estimate the genomic makeup of *Iris nelsonii*, developed a genetic linkage map from a cross between *I. nelsonii* and one of its progenitor species, *I. hexagona* to investigate genomic collinearity, determined the response of each species to abiotic habitat conditions, described the pollination biology of *I. nelsonii*, and investigated the genetic architecture of floral differences that may contribute to pollinator isolation in this system. *Iris nelsonii* appears to be genotypically intermediate between at least *I. fulva* and *I. brevicaulis*, but a larger number of molecular markers are needed in order to thoroughly investigate the hypothesized hybrid origin of *I. nelsonii*. The genome of *I. nelsonii* appears to be roughly collinear with the other species of Louisiana Iris, suggesting that reproductive isolation is not due to chromosomal rearrangements. Ecological isolation is largely important in this system as the species of Louisiana Iris appear to respond to abiotic habitat conditions and *I. nelsonii* and *I. fulva* are visited by different pollinators than *I. hexagona* and *I. brevicaulis*. Despite the originally strict early verbal models of hybrid speciation, reproductive isolation between a hybrid lineage and progenitors can be a result of barriers other than chromosomal rearrangements and is likely a function of a number of ecological, genic, and/or chromosomal barriers.
CHAPTER I: INVESTIGATING THE PURPORTED HOMOPLOID HYBRID ORIGIN OF IRIS NELSONII

Abstract

Hybridization between diverging taxa may result in the production of a novel hybrid lineage that is reproductively isolated from the originally hybridizing taxa. Many species have been hypothesized to have resulted from homoploid hybrid species as a result of their intermediate morphology. The development of molecular markers has enabled the testing of such hypotheses. When the species was originally described, Iris nelsonii was hypothesized to be of hybrid origin based on intermediate morphology and the presence of marker chromosomes of multiple widespread species of Louisiana Iris. Here, I utilized nuclear microsatellite markers to investigate whether I. nelsonii is indeed a distinct species and whether this species is of hybrid origin. Bayesian clustering analysis of sampled individuals of each species suggests that I. nelsonii is indeed a distinct species on an independent evolutionary trajectory from the other species. A principal coordinates analysis of the genetic data suggests that I. nelsonii genotypes are intermediate between the purported progenitor species in multidimensional space, providing support for a hypothesized hybrid origin of I. nelsonii. However, Bayesian clustering analysis of the same data was ambiguous on the genomic makeup of I. nelsonii, suggesting that more
nuclear markers are needed in order to determine the contribution of each widespread species to the genome of *I. nelsonii*.

**Introduction**

While a strict interpretation of the biological species concept requires that no naturally occurring hybrids are produced between ‘good’ species (Mayr 1942), in reality, reproductive isolation is often not complete between closely related species. This is to be expected, as species divergence is a continuous process and the evolution of complete isolation is not expected to be ‘instantaneous’ except in unusual circumstances (e.g. polyploidy). Although most hybrids between divergent taxa reveal reduced fitness relative to their parental progenitors, some hybrid individuals have been shown to be equally or more fit than pure species (Arnold and Hodges 1995). This higher hybrid fitness may facilitate constructive outcomes of hybridization (e.g. adaptive trait introgression and speciation) which are increasingly documented in both plants and animals (reviewed in Arnold 1997, 2006; Rieseberg 1997; Mallet 2007; Mavarez and Linares 2008). One potential outcome of hybridization between species is the formation of new hybrid lineages that are reproductively isolated from their progenitors, either with or without a change in chromosome number.

Hybrid lineages that result from a doubling of chromosome number (i.e. allopolyploids) are often reproductively isolated from the progenitors quickly upon formation (Coyne and Orr 2004). The change in chromosome number may cause physiological changes in the polyploid that translate into ecological or phenological
divergence (reviewed in Coyne and Orr 2004), ultimately resulting in increased assortative mating within the polyploid lineage. In addition to prezygotic barriers that arise directly as a result of increased dosage in the polyploid, chromosomal doubling results in strong postzygotic isolation between the polyploid and its progenitors because triploid hybrids are often sterile (Grant 1981; Coyne and Orr 2004; but see Ramsey and Schemske 1998). As such, these lineages are quickly reproductively isolated from their progenitors, though their establishment probabilities will depend on them being spatially, temporally, and/or ecologically isolated from their progenitors (Coyne and Orr 2004).

Homoploid hybrid species, however, may take many more generations to stabilize (Ungerer et al. 1998; Buerkle and Rieseberg 2008), and thus often contain mixed genomic contributions from each of the progenitor species (Mallet 2007; Jiggins et al. 2008). Indeed the contribution of parental regions to the genome of homoploid hybrid species is highly variable, potentially due to the effect of demographic processes, asymmetric reproductive isolation between the progenitors, drift, and/or selection. The genomes of some stabilized hybrid lineages have been shown to reflect large contributions of each progenitor to the hybrid genome (e.g. *Senecio*: James and Abbott 2005; *Lycaenides*: Gompert et al. 2006; *Stephanomeria*: Sherman and Burke 2009). Alternatively, the genomes of other hybrid lineages have been found to be mostly representative of the genome of a single progenitor, with introgressed alleles derived from other progenitor species being important in effecting adaptation and reproductive isolation (e.g. *Heliconius*: Mavarez et al. 2006). This latter mechanism of homoploid hybrid speciation is perhaps different than traditional “mosaic genome hybrid speciation” based on the demographic and selective environment that the hybrid lineage encounters.
during establishment (Jiggins et al. 2008). In homoploid hybrid lineages with unequal genomic contributions, loci that are derived from the “minority progenitors” represent hypotheses for regions involved in adaptive introgression (e.g. Whitney et al. 2006) and reproductive isolation (e.g. Mavarez et al. 2006). Such hybrid species hold clues about the genetic architecture of speciation in sympatry but are potentially hard to detect because of the need for a large number of molecular markers in order to identify the few regions that are derived from the minority progenitor.

The Louisiana Iris species complex consists of three morphologically distinct, interfertile, and widespread species: *Iris fulva*, *I. brevicaulis* and *I. fulva*. Hybrid zones between these three species have been described where their ranges overlap in southern Louisiana (Arnold 1993). A fourth species, *I. nelsonii*, is locally endemic to a single swamp in Southern Louisiana and is likely ecologically isolated from the three more widely-distributed species (Taylor et al. 2011, 2012). *Iris nelsonii* is morphologically intermediate to, at least, *I. fulva* and *I. hexagona* (Randolph 1966) but lives in a unique highly shaded swamp habitat that is not occupied by the other species. *Iris nelsonii* appears to possess marker chromosomes diagnostic of *I. fulva* and *I. hexagona*, suggesting that this species may be of hybrid origin (Randolph et al. 1961; Randolph 1966).

Randolph (1966) hypothesized that *I. nelsonii* is derived from hybridization between *I. fulva*, *I. hexagona*, and potentially *I. brevicaulis* based on chromosomal and morphologic characteristics (Randolph et al. 1961; Randolph 1966). Molecular evidence has since confirmed that *I. nelsonii* shares both allozyme and RAPD markers with all three purported progenitor species, though *I. fulva* markers are more often represented in
I. nelsonii than I. hexagona and I. brevicaulis markers (Arnold et al. 1990, 1991; Arnold 1993). As such, I. nelsonii has been highly cited as an example of homoploid hybrid speciation, which apparently arose via hybridization and subsequent backcrossing towards I. fulva (e.g. Arnold 1997; Rieseberg 1997; Coyne and Orr 2004). However, the molecular evidence suggesting that I. nelsonii is, in fact, a “good” species is “not yet conclusive” (Abbott et al. 2010), owing to the fact that while all I. nelsonii individuals sampled shared a chloroplast haplotype (Arnold et al. 1991) and high genetic and morphological identity with Iris fulva (Arnold et al. 1990), some of the sampled I. nelsonii individuals in early studies did not in fact share alleles with I. brevicaulis and I. hexagona (Arnold 1993) as would be expected if I. nelsonii is a stabilized homoploid hybrid species.

Here, I utilized a large number of molecular markers to further investigate the purported hybrid origin of I. nelsonii. Specifically, these markers allowed me distinguish between hypotheses for the origin of I. nelsonii and ask the questions 1.) Is I. nelsonii divergent from I. fulva and the other species of Louisiana Iris? and 2.) if so, is I. nelsonii of hybrid origin or is it simply a morphologically divergent population of I. fulva, as some aspects of its floral morphology might suggest?

Methods

A Bayesian assignment approach was used to investigate the hybrid origin of I. nelsonii and estimate of the percentage of the genome that I. nelsonii derived from each of its putative parental species (I. brevicaulis, I. fulva, and I. hexagona). Genotypic data
for these analyses were generated from wild-collected *Iris* plants. Ramets were collected from approximately 30 individuals at three to five sampling localities representative of the ranges of each widespread species in Louisiana in 2007 and 2008. Sampling localities are shown in Figure 1.1 (Taylor et al. 2011). *I. nelsonii* is restricted to a region of Vermillion Parish, Louisiana. As such, *I. nelsonii* was sampled from only this locality.

![Figure 1.1: Iris collecting locations](image)

**Figure 1.1: Iris collecting locations.** *Iris brevicaulis*: Opelousas (Ib.1), Grangeville (Ib.2), Cade (Ib.3); *I. fulva*: BayouCortableau (If.1), Lottie (If.2), Livonia (If.3), BatonRouge (If.4); *I. hexagona*: PinelIslandRd (Ih.1), Esther (Ih.2), LiveOakRd (Ih.3), AveryIsland (Ih.4), Weeks (Ih.5); *I. nelsonii*: TurkeyIsland (In.1). Taken from Taylor et al. 2011.

DNA was extracted from leaf tissue using a modified CTAB DNA extraction protocol. Genetic variation in *I. nelsonii* and the purported progenitor species was investigated at 17 Iris EST-derived microsatellite loci (developed by Tang et al. 2009).
Each sampled locus was chosen from a different linkage group in order to sample as much of the genome as possible with the limited number of markers. PCR reactions were conducted as in Taylor et al. 2012b with a protocol modified from Tang et al. (2009). Each reaction tube included 1x PCR buffer, 2.5mM MgCl₂, 0.3 mM of each dNTP, 4pmol fluorescently labeled forward primer (either 6-FAM/HEX/TAMRA), 4pmol reverse primer, 0.5 units GoTaq Flexi DNA Polymerase (Promega Corporation, Madison, WI, USA), and approximately 10 ng of genomic DNA. The thermocycling program was set in a touchdown format to reduce nonspecific amplification (Don et al. 1991).
Thermocycling conditions were as follows: initial denaturation was at 94°C for 1 minute followed by 6 cycles of: 94°C for 30 seconds, 64°C (decreasing in 1°C increments each cycle to 58°C) for 30 seconds, 72°C for 30 seconds, then 33 cycles of: 94°C for 20 seconds, 58°C for 20 seconds, 72°C for 30 seconds with a final extension period of 72°C for 15 minutes (Tang et al. 2009). In order to reduce genotyping costs, reaction products from PCR that utilized different fluorescent labels were multiplexed before being run on an ABI 3700xl capillary sequencer (Applied Biosystems, Foster City, CA, USA) at the Georgia Genomics Facility. Genotypes were scored by eye in GeneMarker v.1.8 (Softgenetics LLC, State College, PA, USA).

Summary statistics, including the percentage of polymorphic loci and heterozygosity, were generated in GenAlEx v6.5 (Peakall and Smouse 2006, 2012). Individual genetic distances were calculated from the multilocus dataset in GenAlEx v6.5 (Peakall and Smouse 2006, 2012) based on the methods of Smouse and Peakall (1999). Variation within the genetic distance dataset was analyzed with a principal coordinates analysis in GenAlEx v6.5 (Peakall and Smouse 2006, 2012).
Genotypic data were further analyzed with the clustering program Structure (Pritchard et al. 2000) which assigned individuals to groups with no knowledge of population of origin. Simulations were run in which the data was assumed to belong to between 2 and 11 clusters (k=2:11). The most appropriate number of clusters in the dataset was determined by the asymptote of log likelihood scores from an average of 10 runs at each k and by the method of Evanno et al. (2005). In order to investigate the hybrid origin of *I. nelsonii* and the proportion of the *I. nelsonii* genome derived from each progenitor, the individuals in the dataset were assumed to belong to three populations (k = 3). The assignment probability produced from such an analysis suggests the genomic contribution of each species to the genome (Pritchard et al. 2000). If the *I. nelsonii* genome is a mosaic of loci derived from all three purported progenitor species, *I. nelsonii* individuals would receive a mixed assignment probability to each of the three clusters that contributed to its genome. However, if *I. nelsonii* was derived from hybridization between only two of the three species, *I. nelsonii* individuals would receive an assignment probability to only those two clusters.

**Results**

A total of 137 individuals were genotyped at 17 loci. All 17 loci were polymorphic and most were highly polymorphic within species (Table 1.1). Other individuals that were originally genotyped were removed from the analysis throughout the process if they had a large amount of missing data or the few instances when the
source pot was obviously mislabeled (e.g. if an *I. brevicaulis* individual appeared
genotypically to be a pure *I. fulva* individual).

**Table 1.1: Summary of genotypic data.** Sample size, number of alleles per locus,
expected heterozygosity (H_e), and the percentage of polymorphic loci for each sampling
locality of each species.

<table>
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<tr>
<th>Species</th>
<th>Sampling locality</th>
<th>N</th>
<th>Mean alleles per locus</th>
<th>Mean H_e</th>
<th>% Polymorphic loci</th>
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<tr>
<td><em>I. brevicaulis</em></td>
<td>Cade</td>
<td>4</td>
<td>3.29(0.32)</td>
<td>0.60(0.06)</td>
<td>94.12%</td>
</tr>
<tr>
<td></td>
<td>Grangeville</td>
<td>15</td>
<td>4.53(0.39)</td>
<td>0.64(0.04)</td>
<td>100.00%</td>
</tr>
<tr>
<td></td>
<td>Opelousas</td>
<td>10</td>
<td>4.59(0.51)</td>
<td>0.62(0.06)</td>
<td>94.12%</td>
</tr>
<tr>
<td><em>I. fulva</em></td>
<td>BatonRouge</td>
<td>10</td>
<td>3.18(0.36)</td>
<td>0.49(0.06)</td>
<td>88.24%</td>
</tr>
<tr>
<td></td>
<td>Cortableau</td>
<td>6</td>
<td>2.65(0.31)</td>
<td>0.45(0.07)</td>
<td>76.47%</td>
</tr>
<tr>
<td></td>
<td>Livonia</td>
<td>16</td>
<td>3.82(0.4)</td>
<td>0.47(0.06)</td>
<td>94.12%</td>
</tr>
<tr>
<td></td>
<td>Lottie</td>
<td>9</td>
<td>3(0.35)</td>
<td>0.49(0.06)</td>
<td>94.12%</td>
</tr>
<tr>
<td><em>I. hexagona</em></td>
<td>AveryIsland</td>
<td>6</td>
<td>2.24(0.28)</td>
<td>0.33(0.07)</td>
<td>70.59%</td>
</tr>
<tr>
<td></td>
<td>Esther</td>
<td>5</td>
<td>2.47(0.33)</td>
<td>0.38(0.07)</td>
<td>76.47%</td>
</tr>
<tr>
<td></td>
<td>LiveOakRd</td>
<td>9</td>
<td>3.35(0.44)</td>
<td>0.48(0.07)</td>
<td>88.24%</td>
</tr>
<tr>
<td></td>
<td>PineIsland</td>
<td>3</td>
<td>2.29(0.29)</td>
<td>0.47(0.08)</td>
<td>70.59%</td>
</tr>
<tr>
<td></td>
<td>Weeks</td>
<td>20</td>
<td>3(0.41)</td>
<td>0.38(0.06)</td>
<td>76.47%</td>
</tr>
<tr>
<td><em>I. nelsonii</em></td>
<td>TurkeyIsland</td>
<td>24</td>
<td>4.82(0.42)</td>
<td>0.55(0.05)</td>
<td>94.12%</td>
</tr>
</tbody>
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A principal coordinate analysis (PCO) of the individual genetic distances
suggested that *I. nelsonii* is genetically intermediate to the three widespread Louisiana
Iris species (Figure 1.4). The three widespread species are differentiated along PC1
(which explained 35.8% of the variation) and PC2 (which explained 21.3% of the
variation). *Iris nelsonii* individuals appear intermediate to *I. fulva* and *I. brevicaulis* on PC1 and intermediate to all three species on PC2.

![Figure 1.2: Principal Coordinates Analysis based on individual genetic distance calculations as implemented in GenAlEx (Peakall and Smouse 2006, 2012). Populations of *I. brevicaulis* (3) are represented by filled shapes; populations of *I. fulva* (4) are represented by greyed shapes; populations of *I. hexagona* (5) are represented by empty shapes; the *I. nelsonii* population from Turkey Island (1) is represented by the greyed outlined circles.](image)

Both methods used to determine the appropriate number of clusters in the data suggested four clusters, corresponding to the four species. All 10 independent runs at k=4 distinguish between the four different species, suggesting that *I. nelsonii* is differentiated from the other species of Louisiana Iris (Figure 1.2).
Figure 1.3: Results of STRUCTURE with the most likely number of clusters (k=4). Individuals are grouped by species as *I. brevicaulis*, *I. fulva*, *I. hexagona*, and *I. nelsonii*.

At k=3, the independent runs did not agree about the genomic makeup of *I. nelsonii* (Figure 1.3). In 5 of 10 runs, *I. nelsonii* was assigned to the same cluster as *I. fulva* (Figure 1.3A). However, in the remaining 5 runs, *I. nelsonii* was assigned to the same cluster as *I. brevicaulis* (Figure 1.3B). With the exception of a few individuals identified as hybrids with k=4, intermediate assignment probabilities were not observed for *I. nelsonii*.

Figure 1.4: STRUCTURE output for k=3 where *I. nelsonii* is assigned to the (A) *I. fulva* cluster and (B) *I. brevicaulis* cluster. Individuals are grouped by species as *I. brevicaulis*, *I. fulva*, *I. hexagona*, and *I. nelsonii*.
Discussion

Plants with intermediate morphology may be hypothesized to be of hybrid origin. However, there are many causes of morphologic intermediacy and genetic evidence has confirmed the hybrid origin of some (Abbott et al. 2000, 2005), but not all (e.g. Spooner et al. 1991) hypothesized hybrid species. A unique Louisiana Iris that inhabits a limited range in southern Louisiana, *I. nelsonii*, is largely morphologically intermediate between two of the more widespread species of Louisiana Iris, *I. fulva* and *I. hexagona* (Randolph 1966). This morphologic intermediacy combined with the presence of both *I. fulva* and *I. hexagona* marker chromosomes in *I. nelsonii* caused Randolph (1966) to hypothesize that this species is of hybrid origin. Early genetic evidence did indeed confirm the contribution of the widespread species to the genome of *I. nelsonii* individuals, but the limited number of markers was not able to differentiate some *I. nelsonii* individuals from *I. fulva* genotypes. As such, early investigations were inconclusive about whether *I. nelsonii* is indeed a distinct species of hybrid origin (Arnold 1993).

A Principal Coordinates Analysis (PCO) of the genetic distances between individuals suggests that *I. nelsonii* is indeed intermediate to the widespread species, but appears closer to *I. fulva* along both axes as was predicted from previous studies. When *I. nelsonii* was described, Randolph (1966) suggested that this species was derived from hybridization between *I. fulva* and *I. hexagona* based on morphology. *Iris nelsonii* individuals are morphologically intermediate between *I. fulva* and *I. hexagona* for many traits (Randolph 1966) where *I. nelsonii* has been referred to as a “super fulva” because it has the floral coloration and general shape of *I. fulva*, but the size of *I. nelsonii* is much larger than *I. fulva* and is much closer to that of *I. hexagona*. Additionally, the response
of *I. nelsonii* to abiotic habitat conditions is most like the response of *I. hexagona* (Taylor et al. 2011). The current analyses do not support the contribution of alleles from *I. hexagona* in the sampled portions of the *I. nelsonii* genome. Contrary to predictions from morphology and the response to abiotic habitat conditions, *I. nelsonii* appears to be genetically intermediate between *I. fulva* and *I. brevicaulis*. Indeed, hybrid zones between *I. fulva* and *I. brevicaulis* have been detected in southern Louisiana (Arnold et al. 1990; Cruzan and Arnold 1993), so this scenario is plausible, despite the substantial difference in flowering phenology between these species (Cruzan and Arnold 1994; Martin et al. 2007). However, in experimental hybrid populations between *I. fulva* and *I. brevicaulis*, the phenotype of *I. nelsonii* was never approximated, even when the hybrid populations were grown in similar conditions as *I. nelsonii* in the field (S. Taylor pers. obs.).

The frequency of homoploid hybrid speciation in nature is unclear, likely because of the difficulty of detecting homoploid hybrids without a large number of molecular markers. Here, we used a sample size consistent with sample sizes used for similar studies in other systems (e.g. Sherman and Burke 2009; Hermansen et al. 2011) to investigate hybrid speciation in the Louisiana Iris complex. However, independent runs of the clustering program STRUCTURE (Pritchard et al. 2000) did not settle on a stable genomic makeup of *I. nelsonii* (Figure 1.3) and the results do not agree with hypotheses based on morphology, suggesting that the current dataset may not contain a sufficient number of informative markers. As previous studies (Arnold et al. 1990; Arnold 1993) suggest that only a small proportion of the *I. nelsonii* genome is potentially derived from *I. brevicaulis* and/or *I. hexagona*, the likelihood of sampling the introgressed loci in a sample of 17 haphazardly chosen microsatellite markers is likely small. As such, we have
developed a next-generation sequencing library of a large number of *I. nelsonii* individuals and geographically close populations of the purported progenitor species in order to most accurately describe the genomic constituents of *I. nelsonii* and determine whether *I. nelsonii* is indeed a homoploid hybrid species or whether it a sister species or subspecies of one of the other species in this complex. If the data do suggest a homoploid hybrid origin of *I. nelsonii*, the increased genome coverage will enable us to determine which loci are derived from *I. fulva* and which loci are derived from *I. brevicaulis* and *I. hexagona*. In conjunction with concurrent genetic mapping studies, this will allow us to determine the role of the introgressed regions in reproductive isolation between *I. nelsonii* and its progenitors.

Acknowledgements

Y. Martin and H. Martin braved the Louisiana swamp to assist in collecting the sampled individuals. M. Arnold and S. Mopper provided collection locations. This work was funded by a Sigma Xi Grant-in-Aid-of-Research and a grant from the American Iris Society Foundation.

Literature Cited


Abstract

Hybrid speciation represents a relatively rapid form of diversification. Early models of homoploid hybrid speciation suggested that reproductive isolation between the hybrid species and progenitors primarily resulted from karyotypic differences between the species. However, genic incompatibilities and ecological divergence may also be responsible for isolation. *Iris nelsonii* is an example of a homoploid hybrid species that is likely isolated from its progenitors primarily by strong prezygotic isolation, including habitat divergence, floral isolation, and post-pollination prezygotic barriers. Here, we used linkage mapping and QTL mapping approaches to investigate genomic collinearity and the genetic architecture of floral differences between *I. nelsonii* and one of its progenitor species *I. hexagona*. The linkage map produced from this cross is highly collinear with another linkage map produced between *I. fulva* and *I. brevicaulis* (the two other species shown to have contributed to the genomic makeup of *I. nelsonii*), suggesting that karyotypic differences do not contribute substantially to isolation in this homoploid hybrid species. Similar to other studies of the genetic architecture of floral characteristics, at least one QTL was found that explained >20% variance in each color

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1 Sunni J. Taylor, Luis D. Rojas, Sheng-Wei Ho, and Noland H. Martin. *Heredity* in press
trait, while minor QTLs were detected for each morphological trait. These QTLs will
serve as hypotheses for regions under selection by pollinators.

Introduction

The evolution of new, reproductively isolated species usually involves the gradual
accumulation of multiple prezygotic and postzygotic reproductive isolating barriers over
time (Coyne and Orr 1989, 1997; Moyle et al. 2004; Malone and Fontenot 2008; Scopece
et al. 2008). An exception to this is hybrid speciation, where reproductive isolation can
evolve quite quickly (James and Abbott 2005; Mallet 2007; Buerkle and Rieseberg
2008). Most commonly, reproductive isolation between a hybrid species and its
progenitors results from postzygotic isolation caused primarily by a change in ploidy
(polyploid speciation), although a growing number of hybrid species are being detected
that are reproductively isolated from their progenitor species without an increase in
ploidy (homoploid hybrid species; reviewed by Rieseberg and Willis 2007).

Reproductive isolation between new homoploid hybrid species and their
progenitors may result from the rapid fixation of chromosomal rearrangements and/or
genic incompatibilities in addition to ecological divergence between the hybrid taxa and
their parental species (Grant 1971; Buerkle et al. 2000, Buerkle and Rieseberg 2008). A
majority of homoploid hybrid species described to date have a mosaic genome (mosaic
geno

ome hybrid speciation; Jiggins et al. 2008), where genomic differences are sorted in
the hybrid genome through both fertility and ecological selection (Karrenburg et al.
2007). In the most well studied of these systems, chromosomal rearrangements play a
substantial role in strong postzygotic isolation between several independently derived
homoploid hybrid sunflower species and their progenitors (Rieseberg 1995; Lai et al. 2005), as was predicted in earlier verbal models of homoploid hybrid speciation (Grant 1971).

Strong reproductive isolation between the homoploid hybrid species and the progenitors may also result primarily from genic differences and ecological divergence (Jiggins et al. 2008). In a few identified homoploid hybrid species (e.g. *Heliconius* butterflies), the introgression of relatively few traits that confer an ecological advantage may be sufficient to cause reproductive (mainly ecological) isolation (hybrid trait speciation; Jiggins et al. 2008; Salazar et al. 2010). Such systems present an opportunity to investigate alternative models of hybrid speciation, especially ones in which genic incompatibilities and ecological isolation are of primary importance in reproductive isolation.

One of the “classic examples” (Coyne and Orr 2004) of homoploid hybrid speciation is that of the Louisiana Iris species *Iris nelsonii*. Randolph (1966) first described this new species and hypothesized a homoploid hybrid origin based on cytological (Randolph et al. 1961) and morphological data (Randolph 1966). Randolph (1966) suggested that *I. nelsonii* was derived from hybridization between two widespread species of Louisiana Iris (*Iris fulva* and *I. hexagona*) and possibly a third widespread species (*I. brevicaulis*). These three species are all found in southern Louisiana but occupy slightly different habitats and display divergent floral phenotypes. *Iris fulva* flowers are relatively small in size, have a copper red color, and have reflexed sepals. The larger *I. brevicaulis* and *I. hexagona* flowers are blue, with prominent nectar guides and stiff sepals. *Iris nelsonii* flowers are dark red in color and morphologically intermediate
between *I. fulva* and *I. hexagona* for some traits, while extreme to the means of the purported progenitors for others (Randolph 1966). The hybrid origin of *I. nelsonii* was later confirmed with allozyme (Arnold et al. 1990) and nuclear (Arnold 1993) data that suggested that a majority of the *I. nelsonii* genome was derived from *I. fulva* with contributions of loci from *I. hexagona* and *I. brevicaulis*.

When Randolph (1966) initially described *I. nelsonii*, he proposed ecological isolation as a major barrier to gene flow between *I. nelsonii* and the progenitors. Indeed, *I. nelsonii* is endemic to interconnected swamp systems in southern Louisiana and responds differently than its progenitors to abiotic habitat conditions (Taylor et al. 2011). In portions of its limited range, *I. nelsonii* is sympatric with one of its progenitors, *Iris hexagona*. These two species occupy similar swamp habitats and respond to abiotic habitat characteristics differently than the other species of Louisiana Iris (Taylor et al. 2011). However, *I. nelsonii* is often found in understory habitats, while *I. hexagona* is found in more open habitats and seems to be limited by shade (Bennett and Grace 1990). Additionally, as is reflected by their suites of floral characters, these species are pollinated by different suites of pollinators. The large blue flowers of *I. hexagona* are primarily visited by bumblebees (Emms and Arnold 2000), while the large red flowers of *I. nelsonii* are primarily visited by ruby-throated hummingbirds (Taylor et al. 2012).

Pollinator isolation, thus, has the potential to be an extremely important ecological barrier to hybridization between *I. nelsonii* and one of its progenitors, *I. hexagona*.

This classic example of homoploid hybrid speciation represents an opportunity to investigate hybrid speciation where postzygotic isolation is potentially minimal between the hybrid species and progenitors and, instead, prezygotic isolation – especially
ecological isolation - is responsible for inhibiting gene flow in the system. Here, we utilize a comparative mapping approach to investigate genomic collinearity between *I. nelsonii* and its progenitors, noting that increased collinearity should be consistent with the high first-generation hybrid fertility observed between these species. Additionally, we investigate the genetic architecture of floral differences between *I. nelsonii* and *I. hexagona* to identify loci potentially under selection by pollinators and responsible for ecological isolation between these taxa.

**Methods**

**Mapping Population**

In order to produce the mapping population used herein, pollen of a wild-collected *I. nelsonii* individual (In10 – collected from Vermillion Parish, Louisiana) was dusted onto the stigmatic surface of a wild-collected *I. hexagona* individual (IhA32 - collected from St. Martin Parish, Louisiana) to produce F₁ hybrid offspring. Flowers from a single F₁ hybrid were self-pollinated to produce the F₂ hybrid mapping population, and ultimately several hundred F₂ hybrid seeds were produced. The F₂ seeds were planted at the University of Georgia greenhouse and monitored for germination success. Successfully germinated seeds were transplanted into six-inch Azalea pots, and repotted into 8-inch Azalea pots. All F₂, F₁, and pure-species plants were transported to the Texas State University greenhouse in 2007 where they have been maintained and transplanted annually into new 10-inch Azalea pots until the present. In all, 281 F₂ plants were used in the genetic map construction described herein.
**Map construction**

DNA was extracted from IhA32, IN10, the F₁, and the 281 F₂ plants using a modified CTAB DNA extraction protocol. A total of 282 microsatellite primers (developed by Tang et al. 2009 for *I. fulva* and *I. brevicaulis* map production) were screened for utility in the *I. nelsonii* x *I. hexagona* F₂ mapping population. Of those, 137 markers were both polymorphic and reliably scored in the mapping population. The marker names reported here are the same as those reported for linkage maps previously constructed using *I. brevicaulis* X *I. fulva* reciprocal backcross populations (Tang et al. 2010). PCR reactions (modified from Tang et al. 2009) were performed in 10µL reaction volumes that included 1x PCR buffer, 2.5mM MgCl₂, 0.3 mM of each dNTP, 4pmol fluorescently labeled forward primer (either 6-FAM/HEX/TAMRA), 4pmol reverse primer, 0.5 units *GoTaq* Flexi DNA Polymerase (Promega Corporation, Madison, WI, USA), and approximately 10 ng of genomic DNA. Loci were amplified using touchdown PCR (Don et al. 1991) to minimize nonspecific amplification. Thermocycling conditions were as follows: initial denaturation was at 94°C for 1 minute followed by 6 cycles of: 94°C for 30 seconds, 64°C (decreasing in 1°C increments each cycle to 58°C) for 30 seconds, 72°C for 30 seconds, then 33 cycles of: 94°C for 20 seconds, 58°C for 20 seconds, 72°C for 30 seconds with a final extension period of 72°C for 15 minutes (Tang et al. 2009). Fragments were multiplexed when possible (when fluorescent labels and/or allele sizes allowed for multiplexing) and run on an ABI 3700xl capillary sequencer (Applied Biosystems, Foster City, CA, USA) at the Georgia Genomics Facility and scored by eye in Peakscanner v.1.0 (Applied Biosystems, Foster City, CA, USA) and GeneMarker v.1.8 (Softgenetics LLC, State College, PA, USA).
Linkage groups were generated in both TMAP (Cartwright et al. 2007) and MAPMAKER 3.0 (Lander et al. 1987; Lincoln and Lander 1992) with LOD≥8 and a maximum distance of 40 cM. Marker order was determined in TMAP (Cartwright et al. 2007). Initially unlinked markers were added to the existing linkage groups at a maximum distance of 45 cM and LOD ≥ 3 using the “near” command in MAPMAKER 3.0. Total map length was calculated by summing the lengths of the linkage groups. Average marker spacing and map coverage was estimated as in Fishman et al. (2001). Genome length was calculated using two methods. First, the genome length was estimated by adding the length of an average marker interval to each end of each linkage group and summing the lengths of the linkage groups. Second, the genome length was estimated as in method 4 of Chakravarti et al. (1991). Map coverage was calculated separately for each of these genome length estimates.

Transmission ratio distortion

Regions of transmission ratio distortion (TRD) are potentially important for preventing (or favoring) locus-specific gene flow between *I. nelsonii* and *I. hexagona* when interspecific pollination occurs between the two species. Deviations from expected Mendelian segregation ratios (1AA:2Aa:1aa) in the F2 generation were analyzed for each microsatellite marker by $\chi^2$ analyses (2 df). For those loci that significantly deviated from 1:2:1 expectations, we further explored for transmission bias (i.e. whether *I. nelsonii* or *I. hexagona* homozygotes were overrepresented at each locus), using $\chi^2$ analyses (1 df).
*Flower color and morphology*

Morphological characters potentially responsible for differential pollinator attraction were measured in the Texas State University greenhouses during the spring of 2009. The total length of the sepal, the length of the sepal blade and sepal stalk, width of the sepal, and flower stalk height were measured on the first flower of each plant on the second day that the flower was fully opened.

*Iris nelsonii* and *I. hexagona* flowers differ in multiple aspects of color, with *I. nelsonii* flowers being dark red and *I. hexagona* flowers being blue. The concentration of anthocyanin pigments in a single petal of each flower was estimated based on absorbance (Wilken 1982). Petals were used instead of sepals as they do not have nectar guides, and color is relatively uniform throughout the entirety of the petal. Anthocyanins were extracted from one pre-weighed petal using acidified methanol (1% w/v HCl in methanol). A subset of individual samples (including red, blue, and hybrid flowers) was screened between 400 and 800 nm on a Biomate 3 UV-Vis spectrophotometer (Thermo Fisher Scientific MA, USA). All samples in this subset revealed a maximum absorbance at 537nm, so absorbance of the full set of samples was measured only at 537nm. Absorbance values were divided by the weight of the petal to calculate the concentration of anthocyanin pigment (Wilken 1982). These species also differ with respect to nectar guide area. *Iris nelsonii* flowers are generally devoid of a nectar guide, while *I. hexagona* flowers display a prominent nectar guide on each sepal. The length and width of the nectar guide of the pure species and F2 plants were both measured in ImageJ (Rasband 2010). As *Iris* nectar guides are roughly triangular, the area of the nectar guide was calculated as the area of a triangle.
Phenotypic correlations between traits may potentially result from genetic correlation owing to pleiotropy or tight linkage between genes. Phenotypic correlations in the F₂ mapping population were estimated for all pairwise trait combinations. The significance of each phenotypic correlation was assessed after sequentially rejective Bonferroni tests (Holm 1979).

Quantitative Trait Locus (QTL) analyses

Genomic regions associated with variation in floral characteristics were detected by Composite Interval Mapping (CIM; Zeng et al. 1994) in Windows QTL Cartographer version 2.5.10 (Wang et al. 2011) using forward and backward regression with the program’s default settings (2cM intervals, 10cM window size, 5 control markers). A genome-wide significance threshold was set for each trait after 1000 permutations of the data (Churchill and Doerge 1994; Doerge and Churchill 1996). A drop below the permutation threshold or a change in the direction of the additive effect was used to distinguish among QTLs on the same linkage group.

Results

Linkage map

All except four markers coalesced into 22 linkage groups (Figure 2.1). The remaining markers were unlinked at the minimum criteria set for linking unlinked markers (LOD ≥ 3, maximum distance 45cM). For convenience, the linkage groups detected in this mapping population are named to correspond with the linkage groups in
Tang et al. (2010). These groups corresponded with 20 of the 21 linkage groups detected in reciprocal backcross linkage maps developed for the closely related *I. fulva* and *I. brevicaulis* species (Tang et al. 2010). The linkage map produced by Tang et al. (2010) is denser than the current map (average 4.6 cM intervals between markers in Tang et al. (2010) versus the 12.4 cM intervals in this map). The reduced density of the current map is attributed to the fact that the microsatellite markers used in this and the Tang et al. (2010) study were developed from *I. brevicaulis* and *I. fulva* individuals and some of these loci did not amplify in the *I. nelsonii x I. hexagona* population or were not variable between the mapping parents (IhA32 and In10). As this map contains fewer loci than the map produced by Tang et al. (2010), including markers necessary to link distal ends of linkage groups, some of the larger linkage groups detected by Tang et al. (2010) were split into smaller “unlinked” linkage groups by the mapping programs with this population. Markers from the ends of linkage groups 1, 2, and 7 respectively grouped together (Figure 2.1), yet because of the large recombination distance between the ends of these linkage groups, they did not link together in the current map. As such, the portions of the current linkage groups are labeled with “a” and “b” in Figure 1. As the markers from the “a” and “b” segments are not linked, the orientation of the segments in relation to each other cannot be determined. The placement of the segments in Figure 2.1 is one interpretation of the possible placement of these segments. No markers from the relatively small linkage group 20 from Tang et al. (2010) were amplified in this *F₂* population. The sum length of this *I. nelsonii x I. hexagona* linkage map was 1379.9 cM, with an average marker spacing of 12.4 cM. The estimated genome length was calculated two ways, which yielded similar results. If calculated by adding twice the marker interval
spacing to each linkage group, the genome length was estimated as 1926.9cM. If calculated as method 4 from Chakravarti et al. (1991), the genome length was estimated as 1948.8cM. Each estimate of genome length was used in calculations of genome coverage. Based on these calculations, approximately 74% of the genome is within 10cM of a marker.

Transmission Ratio Distortion

TRD was observed for approximately one-third of the marker loci (Figure 2.2). Some markers were difficult to genotype with certainty. These markers were re-coded to reflect that uncertainty, which results in a potential loss of genetic information, (e.g. for some individuals, it was difficult to distinguish with certainty among homozygotes and heterozygotes for the *I. nelsonii* allele [to use an example] – yet it was clear that the individual was not homozygous for the *I. hexagona* allele. These individuals were all coded as a separate category [essentially as “not homozygous for *I. hexagona*”] recognized by the mapping programs). Segregation at these loci was investigated under the simplifying assumption that approximately half of the re-coded loci were homozygotes and half were heterozygotes. If this investigation resulted in a substantial change in the TRD (and p-value), the marker was designated with a caret (^) on the linkage map and TRD figures (Figure 2.1 & 2.2). No directional bias in transmission ratio distortion was observed. Of the markers that revealed significant TRD and uncertain genotypes did not affect the interpretation of the results, homozygotes for the *I. nelsonii* allele were overrepresented for 13 markers, while homozygotes for the *I. hexagona* allele were overrepresented in 14 markers. For the remaining markers, heterozygotes were largely under-represented (10/13 markers had a heterozygote deficiency, Figure 2.2).
Figure 2.1: Observed genotype frequencies of homozygous *I. nelsonii* (squares), *I. hexagona* (circles), and heterozygotes (triangles). The X-axis denotes the genetic distances (cM) along each linkage group. Lines at 0.25 and 0.5 represent Mendelian expected frequencies for homozygotes and heterozygotes, respectively. Markers that deviated from these expectations (p<0.05) are designated with an asterisk (*). Genotype frequencies of markers designated with a caret (^) are not represented because these markers had a large number of undistinguishable genotypes.
Figure 2.2: Linkage map created from an F$_2$ cross between *Iris nelsonii* and *Iris hexagona*. The names of both the markers and linkage groups reflect those used by Tang.
et al. (2010). Markers revealing significant transmission ratio distortion are denoted with an asterisk after the marker name. Markers with a large number of uncertain genotypes that substantially influenced conclusions about distortion are designated with a caret (^). Boxes indicate the 1-LOD confidence interval and lines indicate the 2-LOD confidence interval for Quantitative trait loci (QTLs) associated with variation in phenotypic traits. Filled boxes indicate QTLs at which the *I. hexagona* increased the trait value. Open boxes indicate QTLs at which the *I. hexagona* allele decreased the trait value.

*Flower color and morphology*

A majority of the F$_2$ plants (N=184) flowered during the 2009 flowering season. However, only six pure-species *I. hexagona* and five *I. nelsonii* individuals flowered in the experimental setup during the 2009 flowering season. Due to this reduced sample size of pure-species individuals and lack of information from the mapping parents, QTL effect sizes are only reported as a proportion of the variance explained (PVE) in the F$_2$ population. The morphological measurements made in the greenhouse (Tables 2.1 and 2.2) reflected measurements made in natural populations by Randolph (1966), suggesting that the few pure-species plants that did flower in the greenhouse represent typical samples of the species. The flower stalks of the *I. nelsonii* individuals ($\mu=51.4 \pm 12.14$ cm) were slightly shorter than those of *I. hexagona* ($\mu=64.5 \pm 11.77$ cm; $t = 1.6$, df = 7, $P = 0.15$). One QTL was detected that explained a small percentage of the variance in the F$_2$ population (PVE=0.09; Figure 1, Table 2.1). At this QTL, the *I. hexagona* allele resulted in a decrease in flower stalk height.

In *Iris* flowers, the sepal subtends the anther and stigma and is thus likely important in pollinator attraction. The sepal shape of *I. nelsonii* and *I. hexagona* differ in that the sepals of *I. nelsonii* are reflexed and *I. hexagona* sepals are upright. As the *I. nelsonii* sepal is reflexed, the sepal stalk of the *I. nelsonii* flowers ($\mu=1.84 \pm 0.36$ cm) is
significantly shorter than that of the *I. hexagona* flowers (µ=3.67 ± 0.45 cm; \(t = 6.6, \text{df} = 7, P = 0.0003\)). Three QTLs were detected for sepal stalk length. The *I. hexagona* allele results in an increase in the sepal stalk length at all QTLs detected. The sepal blade of *I. nelsonii* individuals (µ=4.85 ± 0.60 cm) in the greenhouse was also significantly shorter than the sepal blade of *I. hexagona* individuals (µ=6.25 ± 0.51 cm; \(t = 3.8, \text{df} = 7, P = 0.007\)). No QTLs were detected for sepal blade length. The total length of sepals was significantly lower in *I. nelsonii* (µ=6.69 ± 0.29 cm) than in *I. hexagona* (µ=9.92 ± 0.82 cm; \(t = 7.5, \text{df} = 7, P = 0.0001\)). Three of the four QTLs detected for sepal total length were in the expected direction, where the *I. hexagona* allele results in an increase in sepal total length. The *I. hexagona* allele decreases the trait value for the other QTL. The sepal width of *I. nelsonii* flowers (µ=3.27 ± 0.34 cm) was also smaller than *I. hexagona* flowers (µ=4.13 ± 0.47 cm; \(t = 2.9, \text{df} = 5, P = 0.04\)). One QTL was detected for sepal width (Figure 1; Table 2.1). At this QTL, the *I. hexagona* allele decreases the trait mean, which is opposite expectations given the species means.
<table>
<thead>
<tr>
<th>Trait</th>
<th>N</th>
<th>F&lt;sub&gt;2&lt;/sub&gt; mean (SD)</th>
<th>Linkage group</th>
<th>Location (cM)</th>
<th>PVE</th>
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<tr>
<td>Stalk height (cm)</td>
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<td>87.63 (13.96)</td>
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<td>21.4 (6.0-56.4)</td>
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<td>Nectar guide area (cm&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>168</td>
<td>.61 (.47)</td>
<td>LG11</td>
<td>104.9 (84.9-118.9)</td>
<td>0.21</td>
<td>0.307</td>
<td>0.046</td>
</tr>
<tr>
<td>Anthocyanin (OD/mg)</td>
<td>115</td>
<td>0.011 (.0026)</td>
<td>LG2B</td>
<td>17.8 (1-17.8)</td>
<td>0.13</td>
<td>-0.001</td>
<td>-0.001</td>
</tr>
<tr>
<td>Anthocyanin (OD/mg)</td>
<td>115</td>
<td>0.011 (.0026)</td>
<td>LG11</td>
<td>82.3 (72.4-103.9)</td>
<td>0.24</td>
<td>0.002</td>
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</tr>
</tbody>
</table>
Many of the sepal measurements were correlated (Table 2.2). In two cases, this correlation was coupled with overlapping QTLs. The total sepal length and the sepal stalk length are strongly correlated and have overlapping QTLs on LG4 with effects in the same direction (Figure 2.1; Table 2.1). Sepal width and total sepal length are also correlated and QTLs detected on LG4 were overlapping and had effects in the same direction (Figure 2.1; Table 2.1).

**Table 2.2: Spearman’s ρ correlation coefficients for tests of pairwise phenotypic correlations.** Coefficients in bold are significant after a sequentially rejective Bonferroni test. Italicized coefficients were significant before, but not after, correction with the sequentially rejective Bonferroni test. Sample size for each trait is given in the diagonal.

<table>
<thead>
<tr>
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<tr>
<td>sepal stalk</td>
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<tr>
<td>sepal total</td>
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<td>sepal width</td>
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<tr>
<td>anthocyanin</td>
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<td>176</td>
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The species also differ in color, where *I. nelsonii* flowers are red, as is typical for many hummingbird pollinated flowers, and *I. hexagona* flowers are blue – typical of many bee-pollinated flowers. *Iris nelsonii* flowers contained a higher concentration of anthocyanin pigments ($\mu = 0.013 \pm 0.001$ OD/mg) than did *I. hexagona* flowers ($\mu = 0.005 \pm 0.0007$ OD/mg; $t = 10.4$, df = 8, $P < 0.0001$). Two QTLs were detected that had mixed effects on anthocyanin concentration (Figure 2.1; Table 2.1). Also, *I. nelsonii* typically has no, or a very small, nectar guide ($\mu = 0.06 \pm 0.06$ cm) while the nectar guide
on *I. hexagona* (µ=64 ± .15 cm) is prominent (*t* = 7.0, df = 9, *P* < 0.0001). Two QTLs were detected that were associated with variation in nectar guide. As predicted by the species mean difference, the *I. hexagona* allele increased the trait mean at both QTLs.

**Discussion**

*Genomic collinearity*

In order for new homoploid hybrid species to arise and persist, they must evolve reproductive isolation from their progenitor species at a relatively early stage. Early verbal models of homoploid hybrid speciation invoked chromosomal rearrangements and the resulting infertility of F₁ hybrids as the most likely mechanism by which new homoploid species arise (Grant 1971). Computer simulations have revealed that, although chromosomal rearrangements play an important role in the establishment of homoploid hybrid species, ecological isolation can greatly increase the degree to which these newly derived species are maintained over time (Buerkle et al. 2000). Indeed, studies in *Helianthus* support chromosomal differences coupled with ecological isolation as a primary mechanism by which repeated homoploid hybrid species have arisen (Rieseberg et al. 1995; Lai et al. 2005). However, hybrid speciation may also be achieved without chromosomal rearrangements if genic incompatibilities and/or ecological divergence isolate the hybrid species from its progenitors (Templeton 1981; Jiggins et al. 2008). The relative importance of karyotypic differences versus genic incompatibilities is not known because there are relatively few studies specifically examining the genomic collinearity of homoploid hybrid species and their progenitors (but see Rieseberg et al. 1995; Lai et al. 2005). While studies in *Helianthus* support chromosomal differences as a largely
important barrier to gene flow (Rieseberg et al. 1995; Lai et al. 2005), a recent study in *Cottus* (Stemshorn et al. 2011) and the current study (Figure 2.3) reveal high degrees of genomic collinearity between the homoploid hybrid lineage and the progenitors, suggesting that other mechanisms are potentially responsible for reproductive isolation between the hybrid lineage and progenitors.

The genomes of all four hybridizing Louisiana Iris species show a high degree of genetic collinearity. Although *I. hexagona* has a different chromosome number than the other three species, interspecific linkage maps between the species reveal little evidence of major chromosomal rearrangements between it and the other three Louisiana Iris species (Tang et al. 2010; Figure 2.3; E. Ballerini et al. unpublished data). The markers in the current study grouped as in maps produced by Tang et al. (2010), and updated by E. Ballerini et al. (unpublished data), from crosses between *I. brevicaulis* and *I. fulva*, with few exceptions. Marker IM192 is the terminal marker of LG 6 in the *I. brevicaulis* x *I. fulva* map, but is 13.8 cM from the top in the current map. Also, on LG 9, marker spacing in the current map is greater than marker spacing in Tang et al. (2010) and marker IM364 is the terminal marker of LG 9 in Tang et al. (2010) but in the middle (72.6 cM) of LG 9 in the current map (Figure 1; Supplementary Figure). Future mapping studies in a cross between *I. nelsonii* and *I. fulva* and between *I. hexagona* and *I. fulva* will allow us to identify the specific order of markers within each of the species. However, the high degree of genetic collinearity observed between these interspecific maps, combined with the fact that F₁ hybrids do not reveal substantial reductions in pollen fertility (*I. fulva* x *I. nelsonii*, *I. nelsonii* x *I. fulva*, and *I. nelsonii* x *I. hexagona* preliminary data shows F₁ fertility is ~85% that of pure species fertility) imply that major
chromosomal rearrangements are not effecting a high degree of postzygotic isolation, lending support to Randolph’s (1966) hypothesis of ecological isolation being primarily responsible for the origin and maintenance of *I. nelsonii* in its unique cypress swamp habitat.
Figure 2.3: Supplemental Figure: Comparison map of Louisiana Iris species. Composite linkage groups derived from Iris brevicaulis x I. fulva backcross mapping populations by Tang et al. (2010) are shown on the left. Linkage groups derived from the Iris hexagona x I. nelsonii F2 mapping population are shown on the right. Lines connect the location of markers. Due to differences in amplification and polymorphism, not all markers from a mapping population are represented in the other mapping population.
Figure 2.3 continued. Supplemental Figure: Comparison map of Louisiana Iris species.

Transmission Ratio Distortion

Non-Mendelian transmission of alleles is routinely reported across a wide variety of interspecific and intraspecific crosses and across a wide variety of taxa (Fishman et al. 2001; Hall and Willis 2005; Bouck et al. 2005; Tang et al. 2010; Koevoets et al. 2011;
Casellas et al. 2012). TRD may result from any number of post-pollination prezygotic (e.g. Fishman et al. 2008), or postzygotic (prior to genotyping) biological processes. Our crossing design could have resulted in some amount of inbreeding depression, as the original parents were wild-collected and they could have been harboring some deleterious alleles in heterozygous form. This could result in an underrepresentation of parental genotypes linked to those deleterious alleles or an overrepresentation of heterozygotes. As such, the TRD observed for loci in which either *I. nelsonii* or *I. hexagona* homozygotes are underrepresented could be caused by postpollination barriers and/or postzygotic processes (including inbreeding depression).

However, for the 13 markers in which TRD was found and the parental genotypes were roughly equal, ten markers revealed heterozygote deficiencies. This pattern cannot be explained by inbreeding depression, and suggests selection against heterozygote individuals at those loci. In reciprocal backcross linkage mapping populations produced between *I. fulva* and *I. brevicaulis* and germinated and grown as seedlings in the same greenhouse as the *I. nelsonii* x *I. hexagona* mapping population roughly one-third of the markers revealed significant TRD (Bouck et al. 2005; Tang et al. 2010). In those same maps, TRD was largely asymmetric, in that *I. fulva* alleles were overrepresented in each genetic background (Tang et al. 2010). Indeed, introgression in natural hybrid populations between these species often shows a pattern of asymmetric introgression of *I. fulva* alleles across species boundaries (Arnold and Martin 2010). However, markers in the current F$_2$ mapping population showed no such asymmetries.
Genetic architecture of floral characteristics

Ecological divergence is important in reducing gene flow between the homoploid hybrid lineage and its progenitors (Buerkle et al. 2000; Gross and Rieseberg 2005). *Iris nelsonii* is likely isolated from at least two of its progenitors (*I. hexagona* and *I. brevicaulis*) by pollinator isolation and differs from all of its progenitors in its unique suite of floral characteristics (Randolph 1966). As a result of these highly divergent floral morphologies, *I. nelsonii* and *I. hexagona* are primarily visited by different pollinator groups. *Iris hexagona* is primarily pollinated by bumblebees (Emms and Arnold 2000) while *I. nelsonii* is primarily pollinated by hummingbirds (Taylor et al. 2012).

The unique floral morphology of *I. nelsonii* is likely due to inheritance of a mixture of loci from the progenitor species. As such, *I. nelsonii* shares some floral characteristics with *I. fulva* and others with *I. hexagona*, but it also has characteristics that are outside of the means of the other species (Randolph 1966). Here, we used QTL mapping to identify loci that differentiate *I. nelsonii* from one of its progenitor species, *I. hexagona*. These loci serve as hypotheses for loci under selection during the formation of *I. nelsonii*. These loci are also likely responsible for maintaining species barriers via pollinator isolation where these species occur in sympatry. Divergent floral morphologies may directly cause reduced interspecific visitation between these two taxa as has been observed between *I. fulva* and *I. brevicaulis* (Martin et al. 2008) and between *I. fulva* and *I. hexagona* (Emms and Arnold 2000).

QTL mapping studies have often found genomic regions that influence variation in multiple floral traits (e.g. Juenger et al. 2000; Fishman et al. 2002; Goodwillie et al.
Pleitropy or tight linkage of QTLs that influence floral traits may constrain floral evolution in a hybrid zone. We detected some colocalization of QTLs for traits in this mapping population. QTLs for sepal traits colocalized on LG4 and LG9. These overlapping QTLs influenced traits for which we detected positive phenotypic correlations (Table 1). Although the remainder of the significant phenotypic correlations is not explained by colocalized QTLs in this map, we caution that many QTLs, especially those of small effect, may remain undetected due to small sample size, as at least half of the phenotypic variance in the F2 population remains unexplained for all traits.

The genetic architecture of floral characteristics in this system is similar to studies examining the genetic architecture of floral characteristics in other species. Here, we detected two QTLs for anthocyanin concentration that together explained a large portion of the variance in the F2 population (total ~37%; Table 2.1). While QTL mapping studies, especially those with limited sample sizes, may tend to overestimate effect sizes (Beavis 1998), a number of other studies that have quantified flower color in mapping populations have generally detected few loci of large effect on the trait as well (e.g. Bradshaw et al. 1995; Bouck et al. 2007; reviewed in Galliot et al. 2006). We also detected two QTLs for nectar guide area that explained approximately 35% of the variation in the trait (Table 2) which is similar to the findings of Bouck et al. (2007) in a cross between the other two Louisiana Iris species (I. brevicaulis and I. fulva). In contrast to floral color differences between species, differences in other aspects of floral morphology appear to be influenced by a larger number of minor QTLs (Fishman et al. 2002; Bouck et al. 2007; Kim and Rieseberg 1999; reviewed in Galliot et al. 2006; but
see Bradshaw et al. 1995). Here, we similarly detected between 0 and 4 QTLs for each morphological trait, with an average of 0.11±0.03 PVE explained by each of these loci.

The large effect of the color loci and the relatively small effect of the morphological loci suggest that the color difference between species may be accomplished with relatively few substitutions, while more mutational steps lay between the divergent morphologies of closely related species (reviewed in Galliot et al. 2006). Understanding the genetic architecture of floral traits and pollinator visitation allows an investigation of the loci that are under selection by pollinators (e.g. Bradshaw and Schemske 2003). The genetic architecture of floral differences and the effect of these differences on pollinator visitation have been studied in few systems. In *Mimulus* and *Petunia*, mutations with large effect on color (carotenoids and anthocyanins, respectively) also have a large effect on pollinator visitation (Bradshaw and Schemske 2003; Hoballah et al. 2007). In analyses of pollinator visitation in experimental arrays of Louisiana Iris, pollinator preference QTLs overlapped with brightness and hue QTLs of relatively small effect in a backcross population between *Iris fulva* and *I. brevicaulis* (Martin et al. 2008). The current mapping population has an advantage over the *I. brevicaulis X I. fulva* mapping population for examining pollinator preferences, because *I. nelsonii* and *I. hexagona* have near-identical flowering phenologies. *Iris fulva* and *I. brevicaulis* have highly divergent flowering times (the peak flowering times of these species are shifted by approximately a month; Martin et al. 2007), which potentially results in experimental arrays that are offered to the pollinators differing throughout the field season, or the “training” of pollinators to prefer certain floral traits over time.
Summary and Conclusions:

We have shown that *I. nelsonii*, a homoploid hybrid, has a genome that is highly collinear with its progenitor species, which comports with the relatively high fertility observed when F$_1$ hybrids are produced between *I. nelsonii* and its parents. This suggests that barriers other than karyotypic rearrangements were largely responsible for the early establishment of this species. Indeed, Randolph (1966) posited that ecological barriers were likely important in reducing gene flow between *I. nelsonii* and its progenitors, and Taylor et al. (2011) have shown that, in fact this hybrid taxon responds differently to abiotic environmental factors than its parental species. This mapping population and the newly-created map presented here will enable us to examine the genetic architecture of ecological divergence between *I. nelsonii* and *I. hexagona*. Pollinator isolation is a potentially strong ecological barrier between *I. nelsonii* and *I. hexagona*, and we are now in the position to perform pollinator array experiments to examine the genetic architecture of pollinator isolation between these species, and to determine whether the genetic architecture of the floral traits examined here reflects that of pollinator isolation.

Acknowledgements

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Literature Cited


CHAPTER III: IDENTIFICATION OF FLORAL VISITORS OF *IRIS NELSONII*

Abstract

Floral visitors of the homoploid hybrid species, *Iris nelsonii*, were observed and identified in order to determine whether *I. nelsonii* is visited by similar floral visitors as its progenitor *Iris* species. The most common floral visitors to *I. nelsonii* were Ruby-throated Hummingbirds which were also largely successful in transferring a pollen dye-analogue between *I. nelsonii* flowers. Other floral visitors included butterflies, wasps, and bee species. The pollinators of *I. nelsonii* have not been previously documented. These results suggest that pollinator isolation may be important in preventing hybridization between *I. nelsonii* and its geographically closest progenitor species, *I. hexagona*.

Introduction

Many closely related plant species express divergent floral characteristics (e.g. color, flower size, inflorescence size, nectar concentration and quantity) and are visited by different pollinator functional groups (Fenster et al. 2004). Natural hybridization between these species may result in hybrids that exhibit parental, intermediate or extreme floral trait values. In many cases, the hybrid flowers are less attractive to potential

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pollinators than pure-species flowers (e.g. Campbell et al. 1997). However, some hybrid flowers may be equally or more attractive to pollinators as the pure species flowers (e.g. Sutherland and Vickery 1993; Emms and Arnold 2000; Wesselingh and Arnold 2000; Martin et al. 2008), or may even potentially attract a new suite of pollinators (Straw 1955). If the hybrid lineage is pollinated by a different suite of pollinators, ethological pollinator isolation may act to prevent gene flow between the hybrid lineage and the originally hybridizing species (Straw 1955; Chase et al. 2010). Here, we observed pollinators of the homoploid hybrid species *I. nelsonii* to determine whether ethological pollinator isolation may operate in this system.

*Iris nelsonii* Rand. (Abbeville Red Iris) is a homoploid hybrid species derived from hybridization between *I. brevicaulis* Raf. (Zigzag Iris), *I. hexagona* Walter (Dixie Iris), and *I. fulva* Ker Gawl. (Copper Iris) (Randolph 1966; Arnold et al. 1990; Arnold 1993). *Iris brevicaulis* and *I. hexagona* produce blue flowers with prominent nectar guides and stiff sepals that are primarily pollinated by bumblebees (Emms and Arnold 2000; Martin et al. 2008). *Iris fulva* flowers are red, have no nectar guides, and have reflexed sepals and are primarily visited by hummingbirds (Emms and Arnold 2000; Wesselingh and Arnold 2000; Martin et al. 2008). *Iris nelsonii* has dark red flowers (Figure 3.1) typical of a hummingbird pollination syndrome, but the primary floral visitors of *I. nelsonii* have not been previously documented.

**Methods**

In order to determine the floral visitors of *I. nelsonii*, we observed floral visitation in two localities within the restricted range of *I. nelsonii* in Vermillion Parish, Louisiana.
Visitation was recorded on April 8 and April 15-17, 2011. *Iris* flowers have three pollination units, each of which is composed of a sepal and a stylar branch subtended by a single anther and the nectary (Figure 3.1). When a pollinator attempts to access the nectar, pollen is deposited on the head or body of the pollinator. When the pollinator visits the next flower, pollen is deposited onto the stigmatic surface that folds down in front of the anther. The shape of the flower is such that visitors can access the reward ‘legitimately’ through the pollination unit (Figure 3.2a) or ‘illegitimately’ by accessing the nectary directly from the side or top of the flower (Figure 3.2b). Visitors were described as 'legitimate' if they visited the pollination unit of flowers in such a way that pollen transfer was possible (Figure 3.2a). Visitors were described as 'illegitimate' if the pollinator attempted to access the nectar or pollen reward without visiting the pollination unit (Figure 3.2b).

**Figure 3.1. Typical Iris nelsonii flower.** *Iris* flowers have three pollination units, each of which is composed of a sepal (A) and a stylar branch (B) subtended by a single anther and the nectary.
In order for a floral visitor, even a ‘legitimate’ visitor, to act as an effective pollinator, it must first pick up pollen from the anthers and transfer that pollen to the stigma of another flower. On April 17, 2011, for a portion of the floral visitors to *I. nelsonii*, pollen transfer success was examined by applying a powder fluorescent dye to the anthers of focal flowers with a paintbrush and noting the success of dye transfer to flowers visited by the potential pollinator once it visited the focal flower. If dye was observed on or very near the stigmatic surface upon initial visual inspection, the visit was considered a successful transfer. If dye was not visible upon initial inspection, the flower was removed and inspected under an ultraviolet light in a dark room. If no dye was found upon further inspection, the visit was considered an unsuccessful transfer.

![Figure 3.2: Example of legitimate visitation (A) and illegitimate visitation (B) to an *I. nelsonii* flower.](image)

**Results**

A total of 67 visiting bouts were recorded during the 4 days of observation, most of which included visits to multiple flowers. Forty-four bouts were classified as legitimate, 18 were classified as illegitimate, 1 bout contained both legitimate and illegitimate visits, and the legitimacy of 3 of the bouts could not be determined. A
majority of the legitimate visits (39/44, 88.6%) were made by *Archilochus colubris* Linnaeus (Ruby-throated Hummingbirds). Of the 39 visits made by hummingbirds, all were classified as legitimate. Two butterfly species, *Danaus plexippus* Linnaeus (Nymphalidae – monarch butterfly) and *Phoebis sennae* Linnaeus (Pieridae – cloudless sulphur), and a black carpenter bee (*Xylocopa* sp.) also made legitimate visits to *I. nelsonii* flowers, though the effectiveness of these insect species as pollen transfer agents was not documented as none of the individuals visited dyed focal flowers. The monarch butterfly made 2 observed legitimate visits and 0 illegitimate visits. Cloudless sulphur butterflies made 3 visiting bouts for a total of 8 flowers and visited 6 of the 8 flowers legitimately. A *Papilio polyxenes* Fabricius (Nymphalidae – black swallowtail) individual appeared to visit legitimately but the observer’s sight line was impaired so the legitimacy of the black swallowtail visit was classified as unknown. Illegitimate floral visitors included a variety of wasps, a hesperiid butterfly (*Thorybes* sp. - Hesperiidae) and a single honey bee (*Apis mellifera* Linnaeus). Fifteen wasp visiting bouts were recorded. In a majority of the recorded visits, the wasps visited the flower illegitimately and robbed nectar. However, for 2 of the 15 visits, sight lines of the observers were impaired so the legitimacy of the visits was classified as unknown.

Ruby-throated Hummingbirds were the only floral visitors that we assayed for pollen transfer success (April 17). Hummingbirds were largely successful at transferring the pollen analogue from the dyed flower to the undyed flower visited immediately after the dyed flower. Of the 10 primary transfer data points that were collected, 8 of the visited flowers received dye, while the remaining 2 flowers did not receive dye. Hummingbirds were also successful at transferring the pollen analogue to subsequent
flowers, although not all subsequently visited flowers were collected to view under ultraviolet light. Of those collected, dye was present on the second and third flowers visited in the same bout, but pollen was not detected on a second flower visited in a different bout.

Discussion

The floral visitors of *I. nelsonii* are largely different from two of its progenitors, *I. brevicaulis* and *I. hexagona* (its geographically closest progenitor). However, *I. nelsonii* shares primary pollinators with *I. fulva*, suggesting that other barriers (e.g. habitat isolation, Randolph 1966) are responsible for isolation between *I. nelsonii* and *I. fulva*. Hummingbirds are largely successful at intraspecific *I. nelsonii* pollen transfer (this study) and intraspecific *I. fulva* pollen transfer (N. Martin unpublished data). However, the two species differ in floral morphology (Randolph 1966) and the ability of hummingbirds to transfer pollen between flowers of these two species is still unknown. Studies of reproductive isolation between *I. nelsonii* and its progenitors are continuing to understand the mechanisms that prevent gene flow between this geographically restricted species and its progenitors.

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Literature Cited


CHAPTER IV: DIFFERENTIAL RESPONSE OF THE HOMOPLOID HYBRID SPECIES, *IRIS NELSONII* (IRIDACEAE), AND ITS PROGENITORS TO ABIOTIC HABITAT CONDITIONS.³

Abstract

Premise of the study: Homoploid hybrid speciation involves the evolution of reproductive isolation between a hybrid lineage and its progenitors without a change in chromosome number. Ecological divergence presumably plays a large role in the stabilization of hybrid lineages, as all homoploid hybrid species described to date are reported to be ecologically divergent from their progenitors. However, the described ecological divergence in most systems is anecdotal and has not been empirically tested.

Methods: We assessed the vegetative response of *Iris nelsonii*, a homoploid hybrid species, and its three progenitor species, *I. brevicaulis*, *I. fulva*, and *I. hexagona*, to different abiotic conditions (i.e. varied sunlight availability and flooding conditions) that largely characterize the habitats of these four species in their natural habitats in Louisiana, U.S.A.

Key Results: The species differed in their responses to the water-level treatment for many of the response variables, including rhizome weight, ramet growth, plant height, and two principle components used to characterize the data. The species differed in their response

to the light-level treatment for root allocation and the principle component used to characterize plant size. *Iris nelsonii* significantly differed from its progenitors, including its most closely related progenitor species, in response to many of the treatments.

**Conclusions:** The differential response to abiotic habitat conditions of *I. nelsonii* suggests that this species is ecologically divergent from its progenitor species.

**Introduction**

Natural hybridization is common in plants (Ellstrand et al., 1996; Whitney et al., 2010a) and has played an important role in the evolution of biodiversity (Arnold, 1997, 2006). Hybridization may commonly lead to the transfer of neutral or adaptive alleles between species (e.g. Kim and Rieseberg, 1999; Martin et al., 2006; Minder et al., 2007; Minder and Widmer, 2008; Gagnaire et al., 2009; Castiglione et al., 2010; Gompert et al., 2010; Whitney et al., 2010b) or diversification by hybrid speciation (reviewed by Mallet, 2007). Many plant species are of hybrid origin, a minority of which are isolated from the progenitor species without a change in chromosome number (i.e. homoploid hybrid speciation; Rieseberg, 1997). Early models of homoploid hybrid speciation stressed the importance of the evolution of intrinsic postzygotic isolation between the hybrid lineage and the progenitor species through either the sorting of chromosomal rearrangements or genic incompatibilities (Grant, 1981). However, modeling experiments suggest that ecological divergence can facilitate homoploid hybrid speciation without substantial intrinsic reproductive isolation between the hybrid lineage and progenitors, and the probability of homoploid hybrid speciation increases with ecological divergence (Buerkle et al., 2000). Indeed, all homoploid hybrid species investigated to date have been
described as ecologically divergent from their progenitors, although many accounts are
still anecdotal and empirical tests of ecological divergence between hybrid species and
progenitors are needed in order to determine the role and nature of ecological divergence
involved in hybrid speciation events (Gross and Rieseberg, 2005).

The Louisiana Iris species, Iris nelsonii Rand., was first hypothesized to be of
hybrid origin based on chromosomal and morphologic characteristics (Randolph et al.,
1961; Randolph, 1966). Molecular evidence later confirmed that I. nelsonii is indeed a
hybrid species with genomic contributions from three widespread species of Louisiana
Arnold, 1993). Genetic analyses suggest that a large proportion of the I. nelsonii genome
is derived from I. fulva (Arnold et al., 1990; Arnold, 1993) with a minority of the genome
originating from I. hexagona and I. brevicaulis. However, morphologically, I. nelsonii
combines traits of all three progenitor species. For example, I. nelsonii rhizomes, leaves,
and flowers are much larger than I. brevicaulis and I. fulva, yet are similar in size to I.
hexagona. Iris nelsonii and I. fulva flowers are both red and characteristic of a
hummingbird pollination syndrome, while I. brevicaulis and I. hexagona flowers are blue
and characteristic of a bee pollination syndrome (Randolph, 1966). This suggests that the
few introgressed regions of I. hexagona and I. brevicaulis may have contributed to
ecological divergence in I. nelsonii.

These three progenitor species are largely allopatric, but they hybridize where
their ranges meet in southern Louisiana (Arnold et al., 1990, 1991; Arnold, 1993; Cruzan
and Arnold, 1993; Johnston et al., 2001a). The widespread species differ in habitat
associations, where I. brevicaulis is commonly found in drier understory habitats, I. fulva
is found in low-elevation sites in both shade and sun alongside rivers and bayous (Viosca, 1935; Johnston et al., 2001a) and *I. hexagona* occurs in vast populations along the coast in full sun and standing water (Viosca, 1935). *Iris nelsonii* is geographically restricted to the deep shade and fluctuating, high water level of the Abbeville swamp (Randolph, 1966). Aspects of the *I. nelsonii* habitat are extreme to the habitats of the progenitors. For example, the water level found in the *I. nelsonii* habitat is higher and more variable than that found in typical *I. fulva* and *I. brevicaulis* habitats (Randolph, 1966) while the light level of the *I. nelsonii* habitat is lower than that of *I. hexagona* populations (Randolph, 1966). Reproductive isolation among the widespread species in the Louisiana Iris species complex is largely prezygotic, (Lowry et al., 2008a; Arnold et al., 2010) with habitat isolation (Johnston et al., 2001; Martin et al., 2005, 2006), reproductive asynchrony (Cruzan and Arnold 1994; Martin et al. 2007), pollinator isolation (Emms et al., 2003; Wesselingh and Arnold, 2000; Martin et al., 2008), and conspecific pollen precedence (Carney et al., 1994; Carney and Arnold, 1997) effecting a large amount of reproductive isolation between these species. The contribution of these common isolating barriers to reproductive isolation between *I. nelsonii* and its progenitors has not been quantified to-date, but preliminary data of fertility and viability of F$_1$ individuals (S. Taylor unpublished data) suggests that reproductive isolation between *I. nelsonii* and its progenitors is also largely influenced by prezygotic barriers.

Abiotic environmental variables such as sunlight (Bennett and Grace, 1990) and water availability (Johnston et al., 2001a,b; Martin et al., 2005; 2006) have been shown to affect the survival, fitness, and persistence of Louisiana Iris species and their hybrids in greenhouse experiments as well as in nature. Hybrid zones between these species are
often mosaics in which different genotypic groups have different ecological (Cruzan and Arnold, 1993) and abiotic (Johnston et al., 2001a) associations, suggesting a role for environment-dependent selection in structuring hybrid zones. Additionally, sun and water availability have been explicitly hypothesized to influence the spatial isolation of \textit{I. nelsonii} (Randolph, 1966). \textit{Iris nelsonii} was reported to grow in deeper shade than either \textit{I. hexagona} or \textit{I. fulva}, and in deeper water than \textit{I. brevicaulis} (Randolph, 1966), suggesting that \textit{I. nelsonii} may outperform the progenitor species under shaded and flooded conditions. Here, we experimentally manipulated shade levels and water availability in a common garden experiment in order to determine whether \textit{I. nelsonii} is divergent from the progenitor species in response to these abiotic conditions.

Methods

\textit{Plant material}

Ramets were collected from multiple populations of the widespread progenitor species in southern Louisiana and one population of \textit{I. nelsonii}. Collecting locations were chosen to represent the range of the species within the state of Louisiana and are shown in Figure 4.1. \textit{Iris fulva} plants were collected from one locality in St. Landry Parish (If.1, \(n=31\)), two localities in Pointe Coupee Parish (If.2, \(n=29\); If.3, \(n=45\)), and one locality in East Baton Rouge Parish (If.4, \(n=26\)). \textit{Iris brevicaulis} plants were collected from one locality in each St. Landry Parish (Ib.1, \(n=53\)), St. Helena Parish (Ib.2, \(n=59\)), and St. Martin Parish (Ib.3, \(n=43\)). \textit{Iris hexagona} plants were collected from three localities in Vermillion Parish (Ih.1, \(n=8\); Ih.2, \(n=12\); Ih.3, \(n=22\)) and two localities in Iberia Parish (Ih.4, \(n=21\); Ih.5, \(n=48\)). The distribution of \textit{I. nelsonii} is restricted to the Abbeville
swamp system in Vermillion Parish in southern Louisiana and the population of *I. nelsonii* is thought to be relatively uniform across its limited distribution (Randolph, 1966). As such, only one population of *I. nelsonii* was sampled (In.1, n=44). In order to increase sample size and the number of sampled populations, our wild collected plants were supplemented with *I. brevicaulis* (n=12) and *I. nelsonii* (n=25) plants that were previously collected from southern Louisiana and maintained in the University of Georgia greenhouse. Plants used in the experiment were maintained in the Texas State University-San Marcos Department of Biology greenhouse facility for longer than one year before use in this experiment.
Figure 4.1 Collection locations in southern Louisiana. Population locations are more fully described in the materials and methods section of the text.

Experimental setup

This experiment was carried out at the Texas State University-San Marcos greenhouse facility. In July 2008, leaves and roots were trimmed to approximately 5 cm from the rhizome and excess rhizome was trimmed. Rhizomes were weighed to account for variation in initial weight and were then planted into 25 cm x 12 cm bulb pots in an all-purpose potting mix. In total, 483 rhizomes were used in the experimental treatments (I. brevicaulis: 167, I. fulva: 131, I. hexagona: 113, I. nelsonii: 69). If available, multiple
ramets per genotype (average: 1.4 ramets/genotype) were used in this experiment, but ramets of the same genotype were not included in the same block to avoid replicating genotypes within blocks. Before initiating the experiment, all plants were kept under a 90% shade cloth (10% ambient) and watered daily for 20 days to allow the plants to establish roots. The cloth was then removed for five days to expose the plants to full sunlight. Plants were then randomly assigned to blocks within the following treatments: shade-flooded, shade-drained, sun-flooded, and sun-drained. Shade treatments were established under shade cloths (10% ambient) that were hung approximately 1.5 m above the plants and covered the sides of the blocks. Sun treatments were exposed to 100% ambient sunlight. The flooded blocks were enclosed in 2.5 m x 2.5 m tanks. Tank walls were 24 cm tall to allow water to cover the soil surface. Plants in the drained treatment blocks were placed on weed-exclusion paper and watered every third day. The flooded blocks were refilled to their original level every three days. Two blocks containing approximately 60 plants each were established for each treatment for a total of eight experimental blocks.

Plant growth response was measured in November 2008. Upon harvest, survivorship was assessed and the number of independent growth points (ramets), rhizome weight, and height of the tallest leaf were recorded. Roots and leaves were trimmed to approximately 5 cm from the rhizome and dried for approximately 48 hours in a drying oven before being weighed.
Data analysis

All data analyses were performed in JMP version 8.0 (SAS Institute Inc., Cary, NC). Initially, a fully saturated ANCOVA model was used to analyze the effect of species, treatment, and initial rhizome weight (covariate) on final rhizome weight, dry biomass, leaf height, and root allocation. However, with a fully saturated ANCOVA model, significant interactions were detected between the covariate (initial rhizome weight) and many of the predictors in the model for all response variables. As such, the residuals from a regression of each response variable against initial rhizome weight were added to the grand mean of the response variable, and this value was used as the corrected response variable in all subsequent analyses. Each adjusted response variable was used in a fully saturated ANOVA model with species, light-level (sun or shade), and water-level (flooded or drained) used as the main effects. Change in ramet number and root allocation were log transformed and final rhizome weight was square root transformed to better meet the assumptions of ANOVA. Post-hoc Tukey HSD tests were used to detect significant differences between species means. A nominal logistic regression was used to determine the effect of species, water-level, light-level, and all interactions between predictors on survival.

In order to characterize plant response in a smaller number of variables, principal components analysis was conducted on correlations between final rhizome weight, change in ramet number, dry root weight, dry leaf weight, and leaf height. The first two principal components captured a large amount of variation in the response variables (80.2%). As such, variation in the principal components scores for the first two principal components was analyzed by the same fully saturated ANOVA model described above.
Results

A majority of plants (~98%) survived until termination of the experiment in November 2008. There was no significant difference in survivorship between species ($\chi^2 < 0.0001$, $p > 0.99$), water-level ($\chi^2 < 0.0001$, $p > 0.99$), or light level treatments ($\chi^2 < 0.0001$, $p > 0.99$).

Final rhizome weight is the total non-root underground weight of all ramets produced by the plant. Averaged over all treatments, *I. nelsonii* had a significantly lower final rhizome weight than its progenitor species (Tukey HSD; $P < 0.05$; Fig. 4.2A). Overall, the mean final rhizome weight was significantly higher in the full sun and flooded treatments (Table 1; Fig. 4.2A). However, the species differed significantly in the magnitude of their response to the different water levels (Table 4.1), where *I. brevicaulis* had the greatest difference in rhizome weight between treatments and *I. nelsonii* had the least difference between flooded and drained means (Fig. 4.2A).

Dry leaf weight and dry root weight were combined into the dry biomass response variable. *Iris nelsonii* did not significantly differ from *I. hexagona* or *I. brevicaulis* in total dry biomass, while *I. fulva* plants revealed the highest biomass over all treatments (Tukey HSD; $P < 0.05$). All species had the highest biomass in the sun and flooded treatments (Table 4.1) but a significant light-level x water-level interaction resulted from a significant difference between light-level means in the flooded treatment (Tukey HSD; $P < 0.05$; Table 4.1).

Ramet production (i.e. # of growth points) is a measure of clonal growth of individual genotypes. Overall, *I. brevicaulis* and *I. fulva* produced the most new ramets
through the duration of the experiment (Tukey HSD; $P < 0.05$; Fig. 4.2B). *Iris nelsonii* produced fewer new ramets during the experiment than did *I. brevicaulis* and *I. fulva*, but more ramets than *I. hexagona* (Tukey HSD; $P < 0.05$; Fig. 4.2B). The species differed in the magnitude of their responses, resulting in a significant species x water level treatment interaction (Table 1; Fig. 4.2B).

The leaf height of *I. nelsonii* plants was not significantly different from the leaf height of *I. hexagona* and *I. fulva* leaves, but all species produced taller leaves than those of *I. brevicaulis* (Tukey HSD; $P < 0.05$; Fig. 4.2C). Leaf height of *I. fulva* plants did not significantly differ between treatments (Tukey HSD; $P < 0.05$; Fig. 4.2C); however, *I. nelsonii*, *I. hexagona*, and *I. brevicaulis* leaves in the flooded treatments were significantly taller than those in the drained treatments (Tukey HSD; $P < 0.05$; Fig. 4.2C).

**Table 4.1: Effect of species, light-level, water-level, and all interactions on variation in growth variables in Louisiana Iris.**

<table>
<thead>
<tr>
<th>Predictor</th>
<th>rhizome weight</th>
<th>dry biomass</th>
<th>Δ ramet number</th>
<th>leaf height</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$F_{dfN,dfD}$</td>
<td>$p$-value</td>
<td>$F_{dfN,dfD}$</td>
<td>$p$-value</td>
</tr>
<tr>
<td>species</td>
<td>$15.34_{3,430}$</td>
<td>&lt;0.0001</td>
<td>$5.91_{3,435}$</td>
<td>0.0006</td>
</tr>
<tr>
<td>Light-level</td>
<td>$91.89_{1,430}$</td>
<td>&lt;0.0001</td>
<td>$112.96_{1,435}$</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Water-level</td>
<td>$254.29_{1,430}$</td>
<td>&lt;0.0001</td>
<td>$214.83_{1,435}$</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Species * light-level</td>
<td>$1.56_{3,430}$</td>
<td>0.1995</td>
<td>$1.91_{3,435}$</td>
<td>0.1265</td>
</tr>
<tr>
<td>species * water-level</td>
<td>$2.79_{3,430}$</td>
<td>0.0402</td>
<td>$2.45_{3,435}$</td>
<td>0.0627</td>
</tr>
<tr>
<td>Light-level * water-level</td>
<td>$82.12_{1,430}$</td>
<td>&lt;0.0001</td>
<td>$68.77_{1,435}$</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Species * light-level * water-level</td>
<td>$2.46_{3,430}$</td>
<td>0.0620</td>
<td>$2.53_{3,435}$</td>
<td>0.0565</td>
</tr>
</tbody>
</table>
Table 4.1 (continued): Effect of species, light-level, water-level, and all interactions on variation in growth variables in Louisiana Iris.

<table>
<thead>
<tr>
<th>Predictor</th>
<th>leaf height</th>
<th>root allocation</th>
<th>PC1</th>
<th>PC2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F_{dfN,dfD} p-value</td>
<td>F_{dfN,dfD} p-value</td>
<td>F_{dfN,dfD} p-value</td>
<td>F_{dfN,dfD} p-value</td>
</tr>
<tr>
<td>species</td>
<td>22.35_{3,43} 0.000</td>
<td>9.43_{3,43} 0.000</td>
<td>10.59_{3,43} 0.000</td>
<td>53.22_{3,43} 0.000</td>
</tr>
<tr>
<td>Light-level</td>
<td>38.74_{3,43} 0.000</td>
<td>54.46_{1,43} 0.000</td>
<td>80.99_{1,43} 0.000</td>
<td>51.82_{1,43} 0.000</td>
</tr>
<tr>
<td>Water-level</td>
<td>64.17_{3,43} 0.000</td>
<td>17.72_{1,43} 0.000</td>
<td>243.01_{1,43} 0.000</td>
<td>19.45_{1,43} 0.000</td>
</tr>
<tr>
<td>Species * light-level</td>
<td>2.05_{3,43} 0.000</td>
<td>3.73_{3,43} 0.0114</td>
<td>2.95_{3,43} 0.0325</td>
<td>1.39_{3,43} 0.2455</td>
</tr>
<tr>
<td>species * water-level</td>
<td>4.68_{3,43} 0.000</td>
<td>0.74_{3,43} 0.0534</td>
<td>2.91_{3,43} 0.0345</td>
<td>4.34_{3,43} 0.0050</td>
</tr>
<tr>
<td>Light-level x water-level</td>
<td>0.37_{3,43} 0.5407</td>
<td>21.09_{1,43} 0.000</td>
<td>63.91_{1,43} 0.000</td>
<td>1.96_{1,43} 0.1624</td>
</tr>
<tr>
<td>Species * light-level * water-level</td>
<td>0.86_{3,43} 0.4626</td>
<td>0.49_{3,43} 0.6899</td>
<td>2.41_{3,43} 0.0668</td>
<td>0.67_{3,43} 0.5711</td>
</tr>
</tbody>
</table>

Root allocation of *I. nelsonii* did not significantly differ from *I. fulva* and *I. hexagona*.

However, all three species allocated less biomass to roots than did *I. brevicaulis* (Tukey HSD; P < 0.05; Fig. 4.2D). Overall, the plants allocated the most biomass to roots in the sun and dry treatments, but the species differed in their responses to the light-level treatments (Table 1; Fig. 4.2D). All species allocated a similar proportion of biomass to roots in the sun treatments, but *I. nelsonii*, *I. hexagona* and *I. fulva* allocated significantly less biomass to roots in the shade treatments (Tukey HSD; P < 0.05; Fig. 4.2D).
Figure 4.2: Effect of water or light-level on growth variables in four Louisiana Iris species. Results are shown only if a significant species x water-level or species x light-level effect was detected. A. Final rhizome weight reported as backtransformed least square means (corrected for initial rhizome weight). B. Change in ramet number, reported as backtransformed least square means (corrected for initial rhizome weight) with in the water-level treatments. C. Leaf height (corrected for initial rhizome weight). D. Root allocation, reported as backtransformed least square means (corrected for initial rhizome weight) in the light-level treatments. Error bars represent 95% confidence intervals.

The first two principal components explained 80.2% of the variance in the dataset. Principal Component 1 (PC1, 60.3%) was positively correlated with all variables, most highly with final rhizome weight (0.53), dry leaf weight (0.53), and dry root weight (0.51), and is thus a measure of overall plant size. Averaged over all treatments, _I. fulva_ and _I. brevicaulis_ plants had significantly higher PC1 scores than _I. nelsonii_ and _I._
hexagona plants (Tukey HSD, P<0.05; Fig. 4.3). All species reached the highest PC1 scores in the sun and flooded treatments (Table 4.1; Fig. 4.3), although the degree to which PC1 scores changed between treatments differed among species (Table 4.1; Fig. 4.3). PC1 scores for the four species did not significantly differ in the shade treatments (Tukey HSD, P<0.05; Fig. 4.3). *Iris brevicaulis, I. fulva, and I. hexagona* plants had significantly higher PC1 scores in the sun treatment than the shade treatment (Tukey HSD, P<0.05; Fig. 4.3). However, *I. nelsonii* PC1 scores did not significantly differ between the sun and shade treatments (Tukey HSD, P<0.05; Fig. 4.3). The species also differed in their response to the water level treatment. In the flooded treatment, PC1 scores for *I. nelsonii* were not significantly different from *I. hexagona* PC1 scores but were significantly lower than those of *I. brevicaulis* and *I. fulva*. In the drained treatment, PC1 scores for *I. nelsonii* were not significantly different from *I. hexagona* or *I. brevicaulis* but were significantly lower than *I. fulva* (Tukey HSD, P<0.05; Fig. 4.3).

**Figure 4.3:** Effect of (A) water-level and (B) light-level on PC1 scores (a measure of overall plant size corrected for initial rhizome weight) in four Louisiana Iris species. Error bars represent 95% confidence intervals.
Principal Component 2 (PC2, 19.9%) was positively correlated with final height (0.76) and negatively correlated with change in ramet number (-0.61) and is thus an estimate of plant architecture. In general, *I. nelsonii* mirrored growth patterns of *I. hexagona*, and showed no significant differences from this species in any treatment (Tukey HSD, P<0.05; Fig. 4.4). Overall, the PC2 scores for *I. hexagona* and *I. nelsonii* were significantly higher than those of *I. fulva* and scores of all species were higher than those of *I. brevicaulis* (Tukey HSD, P<0.05; Fig. 4.4). The average PC2 scores were significantly higher in the shaded and flooded treatments (Table 4.1; Fig. 4.4). The PC2 scores of *I. brevicaulis* and *I. fulva* plants did not differ between water-level treatments. However, the PC2 scores for *I. hexagona* and *I. nelsonii* were significantly higher in the flooded treatment than in the drained treatment (Tukey HSD, P<0.05; Fig. 4.4).

![Figure 4.4: Effect of water-level on PC2 scores (a measure of plant architecture corrected for initial rhizome weight) in four Louisiana Iris species.](image)

Error bars represent 95% confidence intervals.
Discussion

Adaptation to divergent habitats is thought to be the most common example of ecological divergence between a homoploid hybrid species and its progenitors (Gross and Rieseberg 2005). However, the fitness of homoploid hybrid species and their progenitors in the habitats of the hybrid and progenitors has been empirically tested in only a limited number of systems (e.g. Donovan et al., 2010; Ma et al., 2010). *Iris nelsonii* has been described as being ecologically divergent from its progenitors in habitat associations (Randolph, 1966), but this has never been empirically examined. Here, we assayed the effect of abiotic habitat conditions on vegetative growth in *I. nelsonii* and its progenitors and found that the hybrid species did exhibit a different response to the abiotic habitat conditions than did the progenitor species, suggesting some degree of ecological divergence has occurred among these species.

Consistent with other experiments that involved the transplant of Louisiana Iris rhizomes into non-extreme conditions (e.g. Emms and Arnold, 1997; Taylor et al., 2009), survivorship of ramets in the four treatment combinations (sun-flooded, sun-drained, shade-flooded, shade-drained) was high in this study. Although the native habitats of the four Louisiana Iris species are often starkly different from each other, and environmental variables such as canopy cover and soil moisture affect the structure of sympatric populations (Bennett and Grace, 1990; Johnston et al., 2001a), here each species did not outperform the others in the environmental conditions intended to be representative of its native habitat. For example, although *I. brevicaulis* is most often found in partially shaded habitats with varying levels of soil moisture, *I. brevicaulis* plants had higher PC1 scores in the sun treatments than in the other treatments (Fig. 4.3). Indications of
ecological divergence were, however, detected by examining the significant species x water-level interactions for rhizome weight, change in ramet number, and both principle components, as well as in the species x light-level interactions for both root allocation and PC1. For example, although the three progenitor species had significantly higher PC1 means in the sun treatment than in the shaded treatment, mean *I. nelsonii* PC1 scores did not differ between sun and shade treatments. These results suggest that *I. nelsonii* may perform equally well in shaded and full sun environments, but may be more tolerant of shading than the progenitor species. Indeed, the typical habitat of *I. nelsonii* is under a dense canopy. However, road-cuts through the swamp have increased available edge habitat where large clumps of *I. nelsonii* individuals occur and appear to thrive in at least partial sunlight.

Although *I. nelsonii* did not outperform the progenitor species in the shade-flooded treatment for all response variables in this experiment, as might be expected from its habitat associations, this does not necessarily rule out a substantial role for habitat isolation in the stabilization and isolation of *I. nelsonii*. Indeed, ecological divergence has been shown to be very important in the stabilization of *Helianthus* homoploid hybrid species (Karrenberg et al., 2007), but recent reciprocal transplant experiments failed to show greater fitness of the homoploid hybrid *H. deserticola* in its native habitat, at least during one field season (Donovan et al., 2010). Instead, the results of the current experiment may not reflect the true fitness of the hybrid and progenitor species in the hybrid species’ habitat for a number of reasons. First, extreme conditions associated with the habitat of the hybrid species may not have been captured in the experimental setup. For example, the height of water in the Abbeville swamp habitat of *I. nelsonii* can reach
1m during the height of the flowering season (Randolph, 1966); this degree of flooding was not replicated in our greenhouse experiment. Second, although this initial experiment benefitted from testing the effects of individual environmental variables (i.e. sun and water availability) on vegetative growth, the species habitats differ in many other abiotic factors, including soil pH and organic composition, that may have an equal or greater impact on vegetative growth than canopy cover and water level (e.g. Emms and Arnold, 1997; Johnston et al., 2001a). Lastly, and perhaps most importantly, we assayed the response of established rhizomes to the different abiotic conditions. Although Johnston et al. (2003) detected similar germination requirements for *I. fulva* and *I. brevicaulis* and suggested that selection at later life history stages may result in the distribution and different habitat associations detected in population surveys of these two species, these two species are the most ecologically similar of the four Louisiana Iris species and we have not yet assayed the effect of abiotic conditions on *I. nelsonii* and *I. hexagona* at earlier life history stages (e.g. germination and seedling growth). As such, reciprocal transplants from seed may show a clearer pattern of ecological differentiation not detected in this study.

In order for ecological divergence to be important in the stabilization and maintenance of a hybrid lineage, such divergence must result in reproductive isolation between the homoploid hybrid lineage and the progenitors (Gross and Rieseberg, 2005). Indeed, ecological divergence may result in reproductive isolation within a few generations (Hendry et al., 2007), as divergent selection may result in selection against immigrants (e.g. Lowry et al., 2008b), and thus can contribute substantially to prezygotic isolation (e.g. Lowry et al. 2008a; Sobel et al., 2010). Adaptation of *Iris* species to
different habitats not only likely results in large amounts of ecogeographic isolation (as per Ramsey et al., 2003), it also appears to structure sympatric populations (e.g. Cruzan and Arnold, 1993; Johnston et al., 2001a) thereby reducing gene flow between species by spatial isolation because most cross-pollination is nearest-neighbor (Wesselingh and Arnold, 2000). Randolph (1966) noted many plants of the progenitor species occur in close proximity to the edge of the swamps inhabited by *I. nelsonii*, but no plants of the progenitor species were detected in the swamp. This suggests that the swamp habitat of *I. nelsonii* may not be an appropriate germination site for the other species. Indeed, Johnston et al. (2003) found that seeds of *I. brevicaulis*, *I. fulva* and their hybrids all germinated best in a watered (but not flooded) treatment under shade, but that seedlings grew faster in the sun treatment and reached the greatest biomass in dry treatments. As such, reduced germination of immigrant seedlings may contribute to habitat isolation in this system.

The genomic contribution of progenitor species to the genome of hybrid species is variable. The genomes of most homoploid hybrid species described to date is a mosaic of loci derived from the progenitor species (e.g. Ungerer et al. 1998; James and Abbott, 2005; Gompert et al., 2006; Sherman and Burke, 2009). However, the demographic and selective environment may result in the production of hybrid lineages with a majority of the genome derived from one progenitor species and a minority of the genome (including loci potentially responsible for ecologically important traits) derived from the other progenitor species (Jiggins et al., 2008). Preliminary analyses suggest that a large portion of the *I. nelsonii* genome is derived from *I. fulva* with minor contributions from *I. brevicaulis* and *I. hexagona* (Arnold et al., 1990; Arnold, 1993). Despite the large
contribution of *I. fulva* to the genome of the hybrid species, *I. nelsonii* responded differently than *I. fulva* to changes in abiotic habitat conditions in this experiment (Figs. 4.2-4.4). Instead, *I. nelsonii* mirrors the growth form of *I. hexagona* in which these two species allocate growth to taller leaves instead of more ramets (Fig. 4.4). Tall leaf height, reduced vegetative growth, reduced root allocation, and reduced sexual reproduction may be associated with growth in high water levels (Grace, 1989), and, water levels are indeed higher in the habitats of *I. hexagona* and *I. nelsonii* than *I. brevicaulis* and *I. fulva* (pers. obs; Randolph, 1966). As *I. nelsonii* shares a majority of its genome with *I. fulva*, we hypothesize that introgressed regions from *I. hexagona* that result in this different plant architecture allowed colonization of the cypress swamp by *I. nelsonii*. We are currently working to map regions of the *I. nelsonii* genome that are responsible for these traits and to determine whether these loci are derived from *I. hexagona*. It is noteworthy that, although *I. nelsonii* mirrors *I. hexagona* in plant architecture and the ability to grow in relatively deep standing water, the distribution of *I. hexagona* is largely limited by shade (Bennett and Grace, 1990), such that these species do not grow sympatrically. Also, other prezygotic barriers (e.g. pollinator isolation) presumably act to reduce gene flow between these species.

**Future directions**

This greenhouse experiment investigated the effect of water and sun availability on the vegetative growth of four Louisiana Iris species, with the goal of determining the abiotic habitat variables that allowed for *I. nelsonii*, a homoploid hybrid species, to be ecologically isolated from its progenitors. While some amount of ecological differentiation was observed between *I. nelsonii* and the three other widespread species of
Louisiana Iris, long-term reciprocal transplant experiments and habitat surveys will likely be needed to identify the factors most important in limiting the range of *I. nelsonii*. Furthermore, it has been shown that selection for traits involved in ecological divergence can largely influence the fixation of genomic blocks during stabilization of a homoploid hybrid species (e.g. Karrenberg et al., 2007). We are currently working to create QTL mapping populations between *I. nelsonii* and its progenitor species to determine whether traits responsible for ecologically-important traits are under divergent natural selection in the hybrid and parental environments and whether the minority of regions introgressed from *I. brevicaulis* and *I. hexagona* are, in fact, responsible for ecological differentiation between *I. nelsonii* and its progenitors.

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