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Evolution

Diploid hybrid speciation in *Penstemon* (Scrophulariaceae)

ANDREA D. WOLFE*†, QIU-YUN XIANG*‡, AND SUSAN R. KEPHART§

*Department of Plant Biology, The Ohio State University, Columbus, OH 43210-1293; and §Department of Biology, Willamette University, Salem, OR 97301

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ABSTRACT **Hybrid speciation has played a significant role in the evolution of angiosperms at the polyploid level. However, relatively little is known about the importance of hybrid speciation at the diploid level. Two species of *Penstemon* have been proposed as diploid hybrid derivatives based on morphological data, artificial crossing studies, and pollinator behavior observations: *Penstemon spectabilis* (derived from hybridization between *Penstemon centranthifolius* and *Penstemon grinnellii*) and *Penstemon clevelandii* (derived from hybridization between *P. centranthifolius* and *P. spectabilis*). Previous studies were inconclusive regarding the purported hybrid nature of these species because of a lack of molecular markers sufficient to differentiate the parental taxa in the hybrid complex. We developed hypervariable nuclear markers using inter-simple sequence repeat banding patterns to test these classic hypotheses of diploid hybrid speciation in *Penstemon*. Each species in the hybrid complex was genetically distinct, separated by 10–42 species-specific inter-simple sequence repeat markers. Our data do not support the hybrid origin of *P. spectabilis* but clearly support the diploid hybrid origin of *P. clevelandii*. Our results further suggest that the primary reason diploid hybrid speciation is so difficult to detect is the lack of molecular markers able to differentiate parental taxa from one another, particularly with recently diverged species.**

Hybridization has long been considered a potential mechanism for plant evolution (1–21). The primary effect of hybridization is an increase in genetic variation, both locally and beyond an obvious hybrid zone (3, 7, 8, 11). Hybridization often results in the formation of ecological races, a critical step in speciation (12). Its importance in polyploid speciation is widely recognized (5, 16, 17), but its evolutionary role has been variously questioned or corroborated over the past 50 years, particularly with respect to diploid hybrid speciation (1–21).

The reticulate nature of angiosperm evolution has been acknowledged from numerous studies supporting organelle capture (22). However, of the estimated 300,000 species of angiosperms, there are <10 clearly documented diploid or homoploid hybrid species based on molecular data (3–5, 6, 9, 18–19, 21). To understand the process of evolution, one first needs the tools to detect the patterns. In the case of reticulate evolution, the necessary tools include morphological, cytological, and molecular characters. Many hypotheses of diploid hybrid speciation are based on morphological and cytological characters (8), but support for this mechanism of evolution has been lacking due to the absence of molecular markers that can clearly distinguish closely related parental species.

The PCR was instrumental in the development of new molecular markers for population studies (23). New methods for generating molecular markers based on PCR include random amplified polymorphic DNA (RAPD), simple sequence repeat (SSR), amplified fragment length polymorphism, and inter-simple sequence repeat (ISSR) techniques. Each of these methods has many advantages as well as limitations (23). For example, RAPD markers are relatively easy to generate but may not be variable enough for some applications or may have problems with reproducibility; SSR markers are extremely powerful for estimating genetic diversity but require a lot of effort for primer development; amplified fragment length polymorphism produces many variable bands but usually requires the purchase of patented kits and specialized equipment; ISSR markers are extremely variable and have proven to be sensitive enough to differentiate cultivars (24–34) but haven't been tested in natural populations.

In this paper, we test the utility of ISSR markers in natural populations by assaying the variability of ISSR banding patterns in a known hybrid complex of *Penstemon* (35–37) where other molecular data were available for comparison (38–40). We demonstrate that the hypervariability of ISSR markers revealed in studies of cultivar differentiation (24–34; reviewed in ref. 23) also occurs in natural populations, and these markers are sensitive enough to thoroughly test hypotheses of hybrid speciation and introgression in contrast to previous studies (38–40).

MATERIALS AND METHODS

Background on Hybrid Complex. Straw (35–37) proposed two classic examples of diploid hybrid speciation in *Penstemon* (Fig. 1). These purported cases of hybrid speciation were hypothesized to have resulted from ethological isolation (35–37), where different classes of pollinators were adapted to different floral morphologies, or as a result of ecological isolation (Fig. 1). Over a series of three studies based on allozymes and restriction-site variation of nuclear rDNA and chloroplast DNA, Wolfe and Elisens (38–40) found numerous markers that separated bird-pollinated *Penstemon centranthifolius* (sect. *Gentianoides*) from insect-pollinated species (sect. *Spectabiles*). However, within sect. *Spectabiles*, there were no allozyme markers and few rDNA and chloroplast DNA (cpDNA) markers to distinguish among species. *Penstemon spectabilis* (purported hybrid derivative I; Fig. 1) lacked additive patterns of rDNA markers of *Penstemon grinnellii* and *P. centranthifolius*, but most populations had some individuals with molecular markers from *P. centranthifolius*. The overall pattern observed did not support a hypothesis of hybrid speciation but was more consistent with introgression of genes from *P. centranthifolius* into *P. spectabilis*. In the second hybrid speciation scenario (purported hybrid derivative II; Fig. 1), *P. spectabilis* and *Penstemon clevelandii* shared most alleles, had identical rDNA profiles, and had only 2–4 cpDNA restriction-

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Abbreviations: ISSR, inter-simple sequence repeat; RAPD, random amplified polymorphic DNA; cpDNA, chloroplast DNA.

†To whom reprint requests should be addressed at: Dept. of Plant Biology, The Ohio State University, 1735 Neil Ave., Columbus, OH 43210-1293. e-mail: wolfe.205@osu.edu.
‡Present address: Dept. of Biological Sciences, Idaho State University, Pocatello, ID 83209-8007.

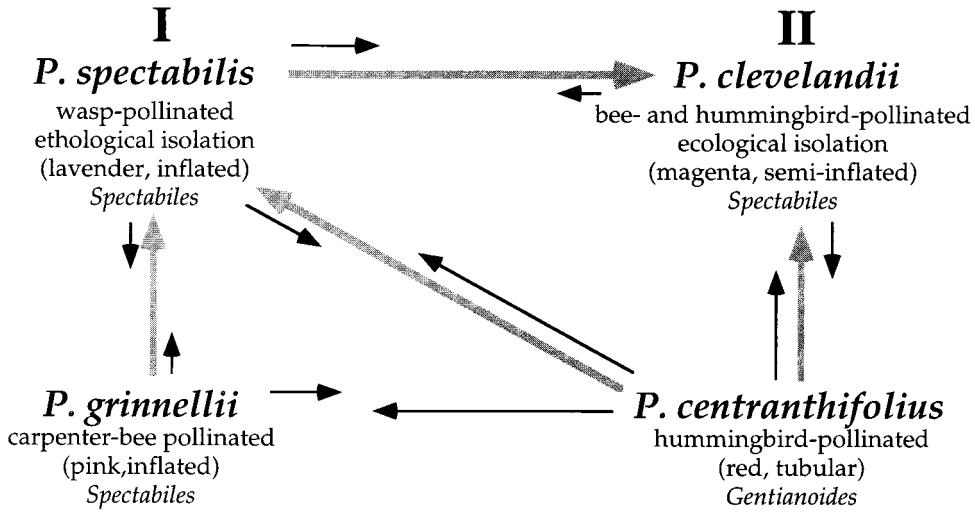

FIG. 1. Schematic of diploid hybrid speciation hypotheses in *Penstemon*. Gray arrows represent directionality of hybrids as proposed by Straw (35–37). Length of the black arrows represent relative degree of gene flow between species supported by four molecular data sets (allozymes, nuclear rDNA and cpDNA restriction-site variation, and ISSR bands). Hypothesized hybrid species I: *P. centranthifolius* has red, tubular corollas and is primarily pollinated by hummingbirds, whereas *P. grinnellii* has pink flowers pollinated by large carpenter bees. The hypothesized hybrid derivative, *P. spectabilis*, purportedly was reproductively isolated from both progenitor species by adaptation to wasp pollination. The second putative hybrid species II, *P. clevelandii*, is primarily pollinated by solitary bees but is also visited by hummingbirds. *P. clevelandii* is adapted to different habitats than its purported progenitors, and ecological isolation is the proposed stabilizing factor in this hypothesis of hybrid speciation. Diploid hybrid speciation hypotheses I and II were supported by morphological similarities of the purported hybrid species with natural and artificial F_1s between the parental taxa and an examination of isolating mechanisms among species in the hybrid complex (35–37). The pattern of molecular markers in the hybrid complex supports the hypothesis of diploid hybrid speciation for *P. clevelandii* but not for *P. spectabilis*, and our results combined with previous studies (38–40) suggest pollen-mediated gene flow between *P. centranthifolius* and both *P. spectabilis* and *P. grinnellii*. We also observed low levels of introgression of ISSR molecular markers between each pair of taxa in section *Spectabiles* in the hybrid complex, except for *P. grinnellii* and *P. clevelandii*.

site differences. *P. clevelandii* had numerous unique alleles, and two of five populations surveyed also shared molecular markers with *P. centranthifolius* but not in a pattern that supported the hypothesis of hybrid speciation. Instead, the overall pattern supported pollen-mediated gene flow from *P. centranthifolius* into *P. clevelandii* and a sister-species relationship between *P. clevelandii* and *P. spectabilis*. Because the few nuclear markers inadequately differentiated the insect-pollinated species of sect. *Spectabiles*, the hypotheses of hybrid speciation could neither be negated nor supported for *P. spectabilis* or *P. clevelandii* (38–40).

Background on ISSR Markers. Simple sequence repeats (microsatellites) are 1–6-nucleotide tandem repeats scattered throughout the genomes of most organisms assayed to date (reviewed in ref. 23). ISSR primers are designed from dinucleotide or trinucleotide simple sequence repeats and are anchored on either the 5′- or 3′-end of the microsatellite with 1–3 nucleotides (23, 25, 27). Where primer matches are found on opposite strands of the DNA molecule within an amplifiable distance, a discrete PCR product results, which can be assayed using nonradioactive or radioactive labeling techniques. ISSR markers are generated by single-primer PCR methods similar to RAPD techniques but have the advantage of exhibiting higher degrees of polymorphism compared with most RAPD markers (23, 28, 32, 33). Each amplicon represents a genetic locus with alleles present or absent. The markers are inherited in a dominant or codominant Mendelian fashion but are scored as diallelic dominant markers for data analysis.

DNA Analysis. One-hundred twenty-two DNA accessions representing 39 populations of species in the hybrid complex were assayed for ISSR polymorphisms (Table 1). Each DNA accession represented one to four individuals and a subset of the DNAs previously assayed using markers from nuclear rDNA and cpDNA restriction-site variation (39, 40).

PCR Amplification and Scoring of Bands. Eight ISSR primers were assayed in single-primer reactions (814.1: $(CT)_8$-TG; 7: $(CT)_8$-RG; 17899: $(CA)_6$-RG; 17898: $(CA)_6$-RY; AW3: $(GT)_6$-RG; 17901: $(GT)_6$-YR; 17902: $(GT)_6$-AY; M1: CAA-$(GA)_{5)}$. Standard reaction conditions were 0.4 μM primer, 1× *Taq* polymerase buffer, 0.2 μM dNTPs, 0.25–0.5 unit *Taq* polymerase (GIBCO/BRL), 3 mM $MgCl_2$, and 0.5 μl DNA in a 25-μl volume. The thermocycle program was 1.5 min at 94°C; 35 × 40 s at 94°C, 45 s at 44°C or 45°C, 1.5 min at 72°C; 45 s at 94°C, 45 s at 44°C, 5 min at 72°C; 6°C soak. PCR reactions were characterized on 1.5% agarose gels in 1× TAE buffer (5 h at 80 mA or until bromphenol blue marker dye migrated 10 cm from origin). Gels were stained with ethidium bromide and were documented digitally using an Alpha Innotech imaging system (Alpha Innotech, San Leandro, CA). The digital image files were transferred in a TIFF format to a PowerMac 7500

Table 1. Distribution and percent polymorphic ISSR bands in *Penstemon* hybrid complex

Parameter	CE	CL	XP	SP	GR
Number of populations	16	5	2	9	7
Number of accessions	46	13	6	31	26
Total bands scored across populations	184	140	108	164	149
Percent bands polymorphic across all populations	95	89	72	90	88
Private bands	42	10	2	15	16
Marker bands	47	3	0	9	8

Private bands are restricted to a single taxon. Marker bands are those found in a relatively higher percentage of one taxon but in a few individuals and populations of other taxa in the hybrid complex (e.g. at least 25% of all populations or individuals in one taxon and only a few individuals in the other taxon). *P. centranthifolius* (CE) is in section *Gentianoides*, whereas *P. clevelandii* (CL), *P. spectabilis* (SP), and *P. grinnellii* (GR) are in section *Spectabiles*. *P.* × *parishii* (XP) is the F_1 hybrid between *P. centranthifolius* and *P. spectabilis*. The close relationship among the species in section *Spectabiles* accounts for the lower numbers of private and marker alleles compared to the more distantly related *P. centranthifolius*.

and analyzed using the BioMax 1D image analysis software (Eastman Kodak). Fragment sizes were estimated based on 1-kb ladder size standards (GIBCO/BRL) according to the algorithm provided in the BioMax 1D software. Fragment sizes were used to assign loci for each primer. Bands were scored as diallelic for each assigned locus (1 = band present; 0 = band absent).

Data Analysis. We calculated the total number of bands and distribution of bands across taxa, number of polymorphic bands, number of fixed bands, and bands shared among taxa. A distance matrix was generated from the raw data matrix of 1s and 0s by using an unpublished computer program written by Vera Ford (University of California, Davis). Pairwise average similarity comparisons between groups of DNA accessions representing specific taxa were calculated.

RESULTS

A total of 270 bands was scored for the five taxa in the hybrid complex. Four bands were fixed across all populations of all taxa (percent polymorphic across five taxa = 98.5). Individual DNA accessions could be genotyped with one to three ISSR primers. Private and marker ISSR bands were found for each species of the *Penstemon* hybrid complex (Table 1) in contrast to previous studies using other molecular data (38–40). The percent polymorphic bands ranged 88–95 for each species and was 72% for the hybrid taxon, *Penstemon* × *parishii* (the F_1 hybrid between *P. centranthifolius* and *P. spectabilis*). Species-specific marker bands and private bands were present in all species, and *P.* × *parishii* had two private bands.

Several marker bands for *P. centranthifolius* were found in some populations of *P. spectabilis*, but only one non-introgressive band (found outside of sympatric and hybridizing populations) was shared exclusively between *P. centranthifolius* and *P. spectabilis* (Table 2) in agreement with patterns previously observed for allozyme and rDNA restriction-site data (38, 39). *P. clevelandii* exclusively shared 15 bands with *P. centranthifolius*, of which 7 were not congruent with a pattern of introgression; 18 bands were exclusive with *P. spectabilis*, with 7 apparently not due to introgression. Patterns for *P. spectabilis* and *P. grinnellii* were nearly identical in regard to bands exclusively shared with *P. centranthifolius* (Table 2, upper and lower diagonals), and the number of bands exclusively shared between pairs of taxa in sect. *Spectabiles*, other than *P. clevelandii*:*P. spectabilis*, were nearly identical.

Average similarity values (Table 3) based on band sharing among populations reveal that *P. spectabilis* and *P. clevelandii* are more similar to one another than *P. spectabilis* is to *P.* × *parishii*, and *P. clevelandii* is more similar to *P.* × *parishii* than *P.* × *parishii* is to its other parental species, *P. centranthifolius*.

Table 2. Patterns of marker bands

	CE	CL	XP	SP	GR
CE		7	5	1	2
CL	8		1	7	5
XP	0	0		3	1
SP	11	1	0		2
GR	8	1	0	6	

Upper diagonal: pattern of marker bands exclusively shared by pairs of taxa in hybrid complex that do not reflect a pattern of introgression as inferred from distribution of four molecular data sets (allozymes, rDNA and cpDNA restriction-site variation, and ISSR markers) across all populations. Introgression was inferred when marker bands were shared among sympatric populations or, in the case of *P. centranthifolius* and *P. spectabilis*, when *P.* × *parishii* also shared a band found in either parental species. Lower diagonal: pattern of marker bands exclusively shared by pairs of taxa in hybrid complex that reflect a pattern of introgression as inferred from distribution of markers in populations and correlation with three other molecular data sets. Abbreviations same as in Table 1.

Table 3. Matrix of average similarity based on bands present that are shared between populations of taxa

	CE	CL	XP	SP	GR
CE	0.529				
CL	0.267	0.533			
XP	0.345	0.457	0.479		
SP	0.277	0.400	0.386	0.522	
GR	0.242	0.370	0.335	0.411	0.504

Average similiarity within each taxon is higher than any pairwise comparison. Abbreviations same as in Table 1.

The average similarity between *P.* × *parishii* and *P. clevelandii* is nearly identical as the average similarity of individuals within *P.* × *parishii*.

DISCUSSION

If *P. spectabilis* were a recent hybrid-derivative of *P. centranthifolius* and *P. grinnellii*, one would expect an additive profile of parental bands in most individuals in all populations of *P. spectabilis*; an ancient hybrid derivative species would likely exhibit additive profiles among individuals in most populations, and a pattern of ancient introgression would show "foreign" markers present among a few individuals in some populations (40). However, populations of *P. spectabilis* lacked an additive profile of bands of *P. centranthifolius* and *P. grinnellii*, exhibited previously observed patterns of introgression of genetic markers from *P. centranthifolius*, and had evidence of low levels of gene flow between the two insect-pollinated species (Fig. 1, Table 2). The combined evidence from allozyme, nuclear rDNA restriction-site variation, and ISSR markers negates the hypothesis of a diploid hybrid origin of *P. spectabilis* but is consistent with pollen-mediated gene flow as proposed by Wolfe and Elisens (40).

Penstemon clevelandii had an additive profile of species-specific ISSR markers and had the highest number of non-introgressive, exclusively shared marker bands with both of its purported parental species (Fig. 1, Table 2). In addition, the average similarity (46%) between *P. clevelandii* and *P.* × *parishii* (the natural hybrid between *P. centranthifolius* and *P. spectabilis*) was higher than *P.* × *parishii* was to either of its parental taxa (35–39%) and was nearly identical to the average similarity within *P.* × *parishii* (Table 3). The morphological similarities between *P. clevelandii* and *P.* × *parishii* include numerous floral and leaf characters (35, 39). *P.* × *parishii* is also commonly found wherever *P. centranthifolius* and *P. spectabilis* occur sympatrically in contrast to the rare occurrence of observed hybrids between the purported progenitors of *P. spectabilis*. Taken together, the propensity for hybridization between the parental taxa, morphological similarities between *P. clevelandii* and *P.* × *parishii*, observed ecological isolation between *P. clevelandii* and its purported parental species, and high genetic similarity between *P. clevelandii* and *P.* × *parishii* all support a diploid hybrid origin of *P. clevelandii* (Fig. 1). However, because *P. clevelandii* has many unique allozyme (38) and ISSR alleles as well as distinct chloroplast haplotypes (40), we suggest an ancient hybrid origin for this species (40).

This study demonstrates that sensitive molecular markers are needed to identify diploid hybrid plant species. Unlike allopolyploid speciation where parental taxa may be highly differentiated, all of the diploid hybrid plant species characterized with molecular data represent hybridization between closely related species, which may be difficult to resolve with standard markers. Although reticulate evolutionary patterns are common in angiosperms, studies using allozymes, restriction-site variation of nuclear and chloroplast genomes, ITS nucleotide sequencing, and RAPD markers have all suggested that diploid hybrid speciation is a rare event in evolution (5, 8,

15, 19, 22, 40). It is clear that numerous species-specific markers for purported parental species are required for documentation of diploid hybrid species. With closely related species, some molecular markers may not offer sufficient variability or consistency to differentiate the parental taxa. However, our results clearly show that in cases where conventional molecular markers fail to reveal patterns of diploid hybrid speciation, hypervariable nuclear markers such as ISSR bands may provide an attractive alternative for assessing whether diploid hybrid speciation is an evolutionary mechanism of importance in angiosperms. Documentation of the diploid or homoploid nature of hybrid species is the first step in understanding the underlying evolutionary mechanisms of hybrid speciation.

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