INTERSPECIFIC HYBRIDIZATION IN NORTH AMERICAN *POLYGALA* (POLYGALACEAE)

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ABSTRACT

As a part of ongoing systematic and phylogenetic studies in Polygalaceae, field collections of two pairs of North American species (*Polygala balduinii* and *P. ramosa,* and *P. lutea* and *P. rugelii*) had morphologically intermediate forms and grew sympatrically, and so were suspected to be interspecific hybrids. Although hybrids among plants are often invoked in taxonomic and floristic literature based on morphologic intermediacy, they are rarely documented and substantiated using molecular tools. We found that the morphologically intermediate individuals within intermixed populations of both species pairs did indeed share all of the variable nucleotide sites in nrITS among the parent species. Likewise, using plastid sequence data (*trn*L-F), we determined that in both species pairs, the parentage was bidirectional. Although some DNA inheritance phenomena (e.g., incomplete lineage sorting) can result in similar polymorphic DNA sequence data, the intermediacy in both morphology and sequence data within sympatric populations is more indicative of interspecific hybridization.

RESUMEN

Como parte de los estudios sistemáticos y filogenéticos en curso de Polygalaceae, las colecciones de campo de dos pares de especies de América del Norte (*Polygala balduinii* y *P. ramosa,* y *P. lutea* y *P. rugelii*) tenían formas morfológicamente intermedias y crecieron de forma simpátrica, y así se sospechó que podrían ser híbridos interespecíficos. Aunque los híbridos entre plantas se invocan a menudo en la literatura taxonómica y florística basada en los intermedios morfológicos, rara vez se documentan y justifican utilizando herramientas moleculares. Encontramos que los individuos morfológicamente intermedios dentro de las poblaciones mezcladas de ambos pares de especies efectivamente compartieron todos los sitios de nucleótidos variables en nrITS entre las especies progenitoras. Del mismo modo, al usar los datos de la secuencia plastidial (*trn*L-F), se determinó que en ambos pares de especies, la filiación era bidireccional. Aunque algunos fenómenos de herencia del ADN (por ejemplo, la clasificación incompleta del linaje) pueden dar como resultado datos de secuencias de ADN polimórficos similares, los intermedios tanto en la morfología como en los datos de secuencias en poblaciones simpátricas es más indicativo de la hibridación interespecífica.

KEY WORDS: interspecific hybrid, nrITS, *Polygala balduinii, Polygala lutea, Polygala ramosa, Polygala rugelii,* Polygalaceae, *trn*L-F

INTRODUCTION

The milkworts (Polygalaceae) are a cosmopolitan family of trees, shrubs, lianas, and herbs. That full variation in life form can be found in the single genus *Polygala* L., as traditionally circumscribed, although most species are herbs. Not surprisingly, phylogenetic analyses have shown that traditional *Polygala* s.l. was an artificial, non-monophyletic assemblage (Persson 2001; Forest et al. 2007; Pastore et al. 2017). Follow-up work has resulted in the re-segregation of historically proposed genera and establishment of a few new ones (Paiva 1998; Castro et al. 2007; Abbott 2009; Pastore et al. 2010; Abbott 2011; Pastore 2012; Pastore & Abbott 2012; Pastore & de Moraes 2013; Abbott & Pastore 2015).

In the course of fieldwork in Florida for broad-scale systematic study of Polygalaceae in North America, four sympatric species were observed to have intermediate morphological forms and, thus were not easily assigned to known species and might represent interspecific hybrids. These same putative interspecific hybrids have been suggested in a taxonomic study of that group of *Polygala* (Smith & Ward 1976). Interspecific hybridization in plants has received a great deal of attention because of its important role in evolution and speciation (Rieseberg et al. 2006; Tate et al. 2006; Mallet 2007, 2008). Some interspecific hybrids have high fitness and have adapted to novel environments relative to their parents (Arnold & Martin 2010), even leading to higher invasive potential for some taxa (Ellstrand & Schierenbeck 2000). Known hybrids are widespread

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around the world in a diverse set of environments (Ellstrand et al. 1996). Given the potential evolutionary significance of hybridization, it is important to document and verify naturally occurring hybrids. Hybridization is a phenomenon commonly invoked historically by taxonomists, yet not frequently substantiated by careful analysis. The purpose of this study was to document the phylogenetic relationships of these species and to assess the putative interspecific hybrid nature of two species pairs based on DNA data (Fig. 1): *P. balduinii* Nutt. with *P. ramosa* Elliott, and *P. lutea* L. with *P. rugelii* Shuttlew. ex Chapm.

MATERIALS AND METHODS

During fieldwork in Florida, sympatric species of *Polygala* were collected that were alongside individuals with intermediate morphology. Those specimens were vouchered for further study using DNA focused techniques to differentiate them. All freshly collected material was preserved in silica gel (Chase & Hills 1991). Genomic DNA was extracted using a modified cetyl trimethylammonium bromide (CTAB) technique (Doyle & Doyle 1987), scaled to a 1 mL volume reaction. Approximately 10 mg of dried tissue were ground in 1 mL of CTAB 2X buffer and 10 µL of proteinase-K. Amplifications were performed using an Eppendorf Mastercycler EP Gradient S thermocycler and Sigma brand reagents in ~25 µL volumes.

nrITS (ITS 1 + 5.8S rDNA+ ITS 2).—This region was amplified with a touchdown protocol using the parameters 99°C, 20 sec; 35X (94°C, 20 sec; 55°C, 20 sec; 72°C, 1 min); 72°C, 2 min with the primers F (TAG AGG AAG GAG AAG TCG TAA CAA) and R (CCC GCC TGA CCT GGG GTC GC) (Hoshi et al. 2008), and with the following reaction components: 0.5 µL template DNA (~10-100 ng), 11 µL water, 7.0 µL 5M betaine, 2.5 µL 10X buffer, 2.5 µL $MgCl_2$ (25mM), 0.5 µL of 10 mM dNTPs, 0.5 µL each of 10 µM primers, and 0.5 unit of *Taq*.

***trn*L-F.—**This region includes the *trn*L intron as well as the *trn*L-F intergenic spacer and was amplified as a single amplicon with the parameters 94°C, 3 min; 30X (94°C, 30 sec; 56°C, 30 sec; 72°C, 1 min); 72°C, 3 min, with the primers C (CGA AAT CGG TAG ACG CTA CG) and F (ATT TGA ACT GGT GAC ACG AG) (Taberlet et al. 1991), and with the following reaction components: 0.5 µL template DNA (~10-100 ng), 17.5 µL water, 2.5 µL 10X buffer, 2.5 µL $MgCl_2$ (25 mM), 0.5 µL of 10 mM dNTPs, 0.5 µL each of 10 µM primers, and 0.5 unit of *Taq*.

PCR products were cleaned with ExoSAP™ (USB Corporation, OH, USA) following the manufacturer's protocols, eluted with 50 µL of 10 mM Tris-HCl (pH 8.5) and stored at 4°C. Purified PCR products were then cycle-sequenced using the parameters 96°C, 10 sec; 25X (96°C, 10 sec; 50°C, 5 sec; 60°C, 4 min), with a mix of 3 µL water, 1 µL fluorescent Big Dye dideoxy terminator, 2 µL Better Buffer™ (The Gel Company), 1 µL template, and 0.5 µL primer. Cycle sequencing products were cleaned using ExoSAP™ following the manufacturer's protocols. Purified cycle sequencing products were directly sequenced on an ABI 3130 automated sequencer according to the manufacturer's protocols (Applied Biosystems, Foster City, CA, USA). Electropherograms were edited and assembled using Sequencher 4.9™ (GeneCodes, Ann Arbor, MI, USA). All sequences were deposited in GenBank (Table 1).

Data analysis.—Sequence data were manually aligned using Se-Al v2.0a11 (Rambaut 1996). No sequence data were excluded from analyses. Indels (insertions/deletions) were not coded as characters. Analyses were performed using PAUP*4.0b10 (Swofford 1999). Fitch parsimony analyses [unordered characters with equal weights; (Fitch 1971)] used a heuristic search strategy which consisted of branch swapping by tree bisection reconnection (TBR), Deltran character optimization, stepwise addition with 1000 random-addition replicates holding 5 trees at each step, and saving multiple trees (MulTrees). Levels of support were assessed using the bootstrap (Felsenstein 1985). Bootstrap percentages under parsimony were estimated with 1000 bootstrap replicates, using TBR swapping for 50 random-addition replicates per bootstrap replicate. Closely related outgroups included *P. nana* (Michx.) DC. and *P. smallii* R.R. Smith & D.B. Ward and were selected based on a densely sampled phylogenetic analysis of Polygalaceae (Abbott 2009).

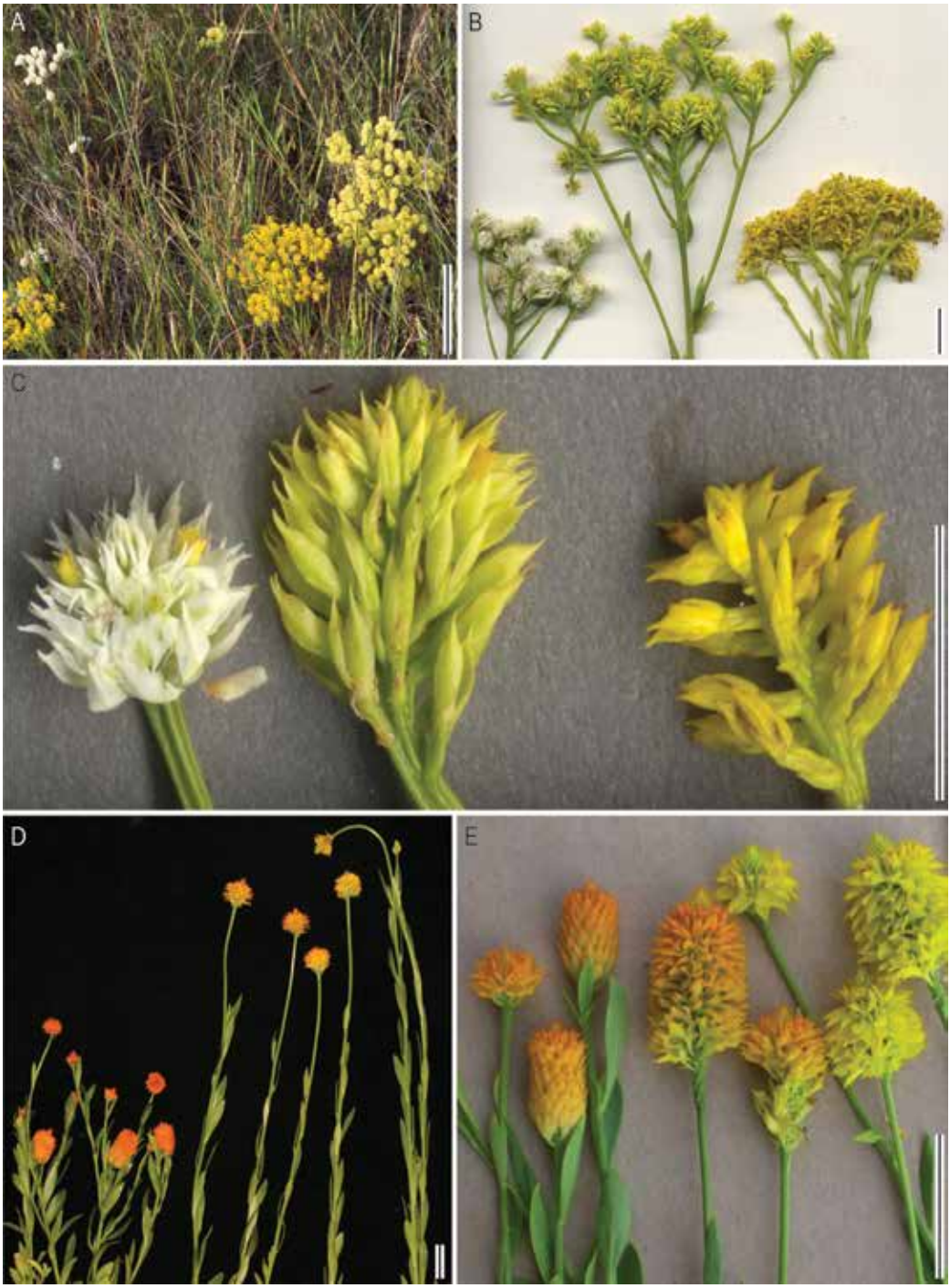

Fig. 1. **Photographs of the parent species and putative hybrids. A.** Habit and habitat of *Polygala balduinii* (white flowers), *P. ramosa* (yellow flowers), and their hybrid (yellowish-green flowers). **B.** Inflorescence structure of these same species and their hybrid. **C.** Inflorescence subunits in detail. **D.** Habit of *P. lutea* (left, *Abbott 24570*), *P. rugelii* (right, *Abbott 24569*), and their hybrid (middle, *Abbott 24571*). **E.** Inflorescence subunits in detail. Photos by J. Richard Abbott.

Table 1. GenBank accession numbers and vouchers used in this study. All vouchers are deposited at the University of Florida herbarium (FLAS), except *Lippincott 40*, which is deposited at Fairchild Tropical Botanic Garden herbarium (FTG).

Species	Collection	ITS GenBank	*trn*L-F GenBank
Polygala balduinii Nutt.	*J.R. Abbott 14972*	KM068813	KM068830
P. balduinii	*J.R. Abbott 14980*	KM068819	KM068836
P. balduinii	*J.R. Abbott 14983*	KM068821	KM068838
P. balduinii	*J.R. Abbott 17824*	KM068823	KM068840
P. balduinii	*J.R. Abbott 14789*	GQ888930	GQ889109, GQ888801
P. balduinii × *P. ramosa*	*J R. Abbott 14973*	KM068814	KM068831
P. balduinii × *P. ramosa*	*J.R. Abbott 14977*	KM068817	KM068834
P. balduinii × *P. ramosa*	*J.R. Abbott 14984*	KM068822	KM068839
P. balduinii × *P. ramosa*	*J.R. Abbott 17826*	NA	KM068841
P. cymosa Walter	*J.R. Abbott 13823*	GQ888947	GQ889126, GQ888815
P. lutea L.	*J.R. Abbott 13632*	KM068811	KM068828
P. lutea	*J.R. Abbott 24570*	GQ888982	GQ888840, GQ889161
P. nana (Michx.) DC.	*J.R. Abbott 8942*	GQ888989	GQ889168, GQ888844
P. ramosa Elliott	*J.R. Abbott 14975*	KM068815	KM068832
P. ramosa	*J.R. Abbott 14976*	KM068816	KM068833
P. ramosa	*J.R. Abbott 14979*	KM068818	KM068835
P. ramosa	*J.R. Abbott 14982*	KM068820	KM068837
P. ramosa	*J.R. Abbott 13640*	GQ889004	GQ889183, GQ888852
P. rugelii Shuttlew. ex Chapm.	*J.R. Abbott 14273*	KM068812	KM068829
P. rugelii	*J.R. Abbott 24569*	KM068826	KM068844
P. rugelii	*J.R. Abbott 22481*	GQ889008	GQ889187, GQ888853
P. rugelii × *P. lutea*	*J.R. Abbott 22712*	KM068824	KM068842
P. rugelii × *P. lutea*	*J.R. Abbott 22906*	KM068825	KM068843
P. rugelii × *P. lutea*	*J.R. Abbott 24571*	KM068827	KM068845
P. smallii R.R. Sm. & D.B. Ward	*C. Lippincott 40*	GQ889021	GQ889200, GQ888861

RESULTS

Putative hybrids all showed intermediate morphology between their parent species. However, the putative hybrids from *P. balduinii* and *P. ramosa* had taller and wider inflorescences than either of the parent species.

Similar intermediacy can be seen in the nrITS sequence data (Fig. 2). Putative hybrid individuals are polymorphic at the same nucleotide positions as each of the parent species. In ITS, *P. balduinii* × *P. ramosa* hybrid individuals had 31 polymorphic nucleotide sites out of 678 in an aligned matrix (Table 2), while *P. lutea* × *P. rugelii* hybrid individuals had 20 polymorphic nucleotide sites out of 719 in an aligned matrix (Table 3). The *trn*L-F data, being a plastid marker, does not show polymorphisms in hybrids. Those matrices were 960 and 876 nucleotides long in an aligned matrix, respectively. These data indicate that each of the parent species has a distinct plastid haplotype, while the putative interspecific hybrids can have either of the two haplotypes of the parents.

DISCUSSION

Based on morphological intermediacy, we observed individuals that seemed to clearly indicate interspecific hybridization between the species pairs: *P. lutea* with *P. rugelii*, and *P. balduinii* with *P. ramosa*. Along with *P. nana* and *P. smallii*, *P. balduinii*, *P. lutea*, *P. rugelii*, and *P. ramosa* form a well-supported clade, in broad scale analyses of Polygalaceae (Abbott 2009), known as *Polygala* ser. *Decurrentes* Chodat. This clade has long been recognized as a natural group based on morphology (Smith & Ward 1976), chromosome number (Lewis & Davis 1962), and distribution within the southeastern US.

We verified our morphological observations using DNA sequence data. Polymorphic sequences of nuclear sequence data can indicate the hybrid nature of individuals or species by indicating retained copies (Ionta et al. 2007; Majure et al. 2012), especially in nrITS (Soltis et al. 2008). Our ITS data showed this pattern

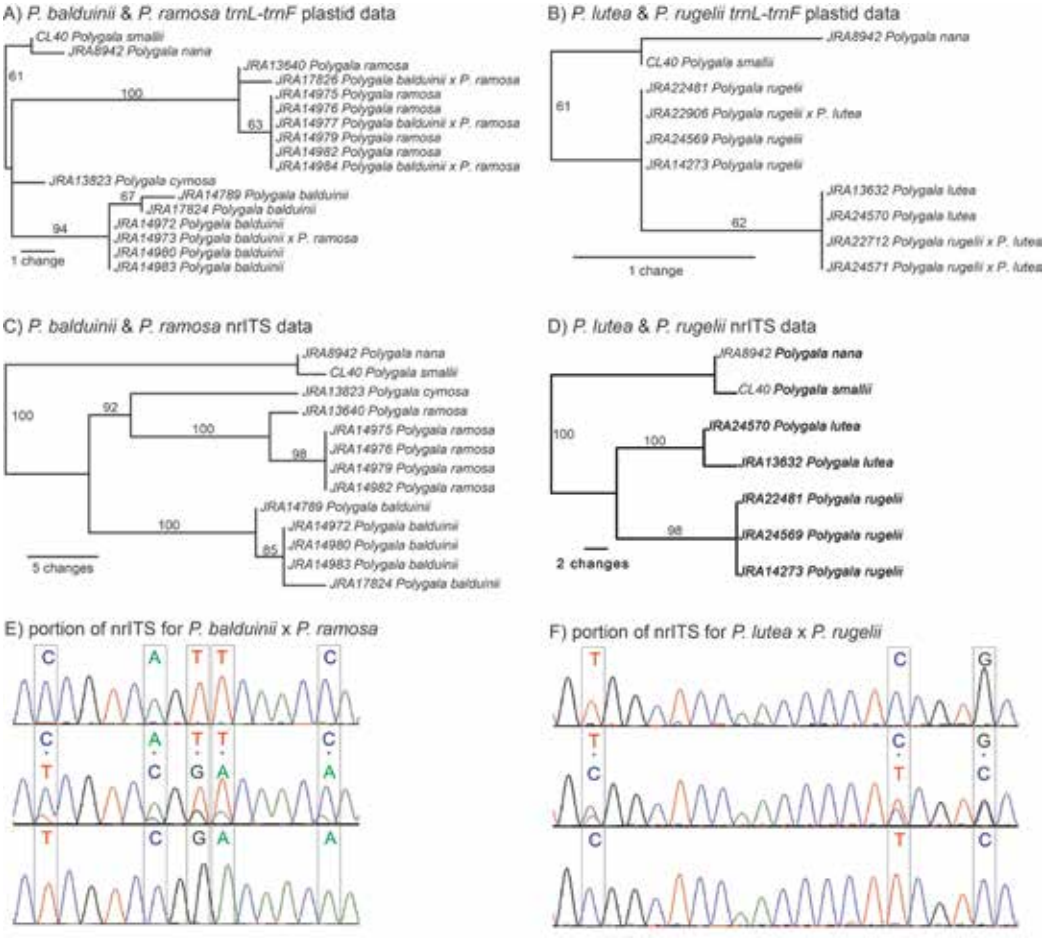

Fig. 2. A–B. Single most parsimonious phylograms based on *trn*L-F sequence data, with bootstrap values. C–D. Single most parsimonious phylograms based on nrITS sequence data, with bootstrap values above branches. Note that individuals with polymorphic sequences (i.e., the hybrids) are not included because of the phylogenetic artifacts associated with hybrids. These phylogenetic trees show levels of pairwise variation between the parent species for this locus. E–F. Portion of electropherograms of nrITS sequence data. Left: *P. balduinii* (*Abbott 14980*) on top, *P. balduinii* × *P. ramosa* (*Abbott 14984*) in the middle, and *P. ramosa* (*Abbott 14982*) on the bottom. Right: P. *lutea* (*Abbott 24570*) on top, *P. lutea* × *P. rugelii* (*Abbott 24571*) in the middle, and *P. rugelii* (*Abbott 24569*) on the bottom.

of overlapping polymorphic nucleotides that coincided in all variable sites compared to the parental species (Fig. 2, Tables 2 & 3).

Within the genus *Polygala* s.l. in North America, reproductive isolation is likely often tied to habitat differences, phenology, and different chromosome numbers (Lewis & Davis 1962). However, most species of ser. *Decurrentes* have been studied and have had the same base chromosome number reported for all, whether n = 32 (Smith & Ward 1976) or n = 34 (Lewis & Davis 1962).

Polygala balduinii and *P. ramosa* share a similar geographic distribution throughout the Gulf coast. While *P. lutea* has a widespread distribution in the eastern United States, *P. rugelii* is endemic to Florida. All of these species can be found growing in close proximity to each other, and it is, in fact, quite common to find 2 or 3 species growing intermixed. As many as 4 species of ser. *Decurrentes* and 6–8 species of *Polygala* s.l. (including

Table 2. A summation of nucleotide sites that differ among *P. balduinii, P. ramosa,* and their putative hybrids. Note that the hybrids are polymorphic at these sites and the representative ambiguity code for those nucleotides is shown.

		Polymorphic site of nrITS																												
		041	070	072	087	099	100	103	104	115	116	162	193	198	200	201	206	252	261	267	285	294	319	515	517	520	526	532	533	566	609	648
Species	**Collection**																															
Polygala balduinii Nutt.	*J.R. Abbott 14789*	G	T	T	C	G	G	T	C	G	C	C	C	C	T	T	C	C	T	A	G	C	A	G	A	C	G	T	G	A	C	T
P. balduinii	*J.R. Abbott 14972*	G	T	T	T	G	G	T	C	G	C	C	C	A	T	T	C	C	T	A	G	C	A	G	A	C	G	T	G	A	C	T
P. balduinii	*J.R. Abbott 14980*	G	T	T	T	G	G	T	C	G	C	C	C	A	T	T	C	C	T	A	G	C	A	G	A	C	G	T	G	A	C	T
P. balduinii	*J.R. Abbott 14983*	G	T	T	T	G	G	T	C	G	C	C	C	A	T	T	C	C	T	A	G	C	A	G	A	C	G	T	G	A	C	T
P. balduinii	*J.R. Abbott 17824*	G	T	T	T	G	G	T	C	G	C	C	C	A	T	T	C	C	T	A	G	C	A	G	A	C	G	T	G	A	C	T
P. balduinii × P. ramosa	*J.R. Abbott 14973*	R	Y	Y	Y	K	K	Y	M	R	M	Y	Y	M	K	W	M	Y	Y	M	R	Y	A	K	W	Y	R	K	K	R	Y	Y
P. balduinii × P. ramosa	*J.R. Abbott 14977*	R	Y	Y	Y	K	K	Y	M	R	M	Y	Y	M	K	W	M	Y	Y	M	R	Y	A	K	W	Y	R	K	K	R	Y	Y
P. balduinii × P. ramosa	*J.R. Abbott 14984*	R	Y	Y	Y	K	K	Y	M	R	M	Y	Y	M	K	W	M	Y	Y	M	R	Y	A	K	W	Y	R	K	K	R	Y	Y
P. ramosa Elliott	*J.R. Abbott 14975*	A	C	C	C	T	T	C	A	A	A	T	T	C	G	A	A	T	C	C	A	T	-	T	T	T	A	G	T	G	T	C
P. ramosa	*J.R. Abbott 14976*	A	C	C	C	T	T	C	A	A	A	T	T	C	G	A	A	T	C	C	A	T	-	T	T	T	A	G	T	G	T	C
P. ramosa	*J.R. Abbott 14979*	A	C	C	C	T	T	C	A	A	A	T	T	C	G	A	A	T	C	C	A	T	-	T	T	T	A	G	T	G	T	C
P. ramosa	*J.R. Abbott 14982*	A	C	C	C	T	T	C	A	A	A	T	T	C	G	A	A	T	C	C	A	T	-	T	T	T	A	G	T	G	T	C
P. ramosa	*J.R. Abbott 13640*	A	C	C	C	T	T	C	A	A	A	T	C	C	G	A	A	T	C	C	A	T	-	T	A	C	A	G	T	A	T	C

TABLE 3. A summation of nucleotide sites that differ among *P. lutea*, *P. rugelii*, and their putative hybrids. Note that the hybrids are polymorphic at these sites and the representative ambiguity code for those nucleotides is shown.

		Polymorphic site of nrITS																					
		0	1	1	1	1	1	1	1	2	2	2	4	4	5	5	5	6	6	6	6	6	6
		5	2	3	3	4	6	7	8	3	4	8	5	8	0	0	5	0	7	8	9	3	5
Species	Collection	8	0	0	9	7	5	9	3	4	4	4	7	4	1	4	3	6	9	3	5		
P. lutea L.	*J.R. Abbott 13632*	T	A	G	G	C	T	C	G	T	T	C	C	A	T	C	G	C	A	C	A		
P. lutea	*J.R. Abbott 24570*	T	A	G	G	C	T	C	G	T	T	C	C	G	T	C	G	C	A	C	A		
P. rugelii × *P. lutea*	*J.R. Abbott 22712*	K	W	R	K	Y	Y	Y	S	W	Y	Y	Y	R	W	Y	K	Y	R	Y	M		
P. rugelii × *P. lutea*	*J.R. Abbott 22906*	K	W	R	K	Y	Y	Y	S	W	Y	Y	Y	R	W	Y	K	Y	R	Y	M		
P. rugelii × *P. lutea*	*J.R. Abbott 24571*	K	W	R	K	Y	Y	Y	S	W	Y	Y	Y	R	W	Y	K	Y	R	Y	M		
P. rugelii Shuttlew. ex Chapm.	*J.R. Abbott 14273*	G	T	A	T	T	C	T	C	A	C	T	T	G	A	T	T	T	G	T	C		
P. rugelii	*J.R. Abbott 24569*	G	T	A	T	T	C	T	C	A	C	T	T	G	A	T	T	T	G	T	C		
P. rugelii	*J.R. Abbott 22481*	G	T	A	T	T	C	T	C	A	C	T	T	G	A	T	T	T	G	T	C		

Asemeia Raf.) have been seen sympatrically in Florida and the adjacent coastal plain, sharing the same seasonally wet open areas (i.e., marshes, flatwoods, wet depressions in sandhills, wet prairies, bogs; Abbott, personal observation), especially when the landscape is a microtopographical mosaic. It is relatively common to find the following taxa, especially, growing intermixed or nearly so: *P. balduinii, P. cruciata* L., *P. cymosa, P. lutea, P. nana, P. ramosa, P. rugelii, P. setacea* Michx., and *Asemeia grandiflora* (Walter) Small. When the other species of *Polygala* in the southeastern United States are encountered, while often a bit rarer in the landscape, they are also often found growing with other *Polygala* species. Yet hybridization, even when there are several abundant members of the ser. *Decurrentes,* is quite rarely encountered, indicating that there must be relatively effective barriers to hybridization. Given all these factors, it is not clear what is driving reproductive isolation, although pollinator differences can be presumed, given floral differences.

In the case of *P. lutea* and *P. rugelii,* these two species are each other's closest relatives. *Polygala balduinii* and *P. ramosa* are very closely related and are also both closely related to *P. cymosa*. Thus, in both of these cases, a shared common ancestry, with the retained ability to interbreed, in conjunction with their sympatry at a local scale, seem to play a role in their likelihood to hybridize.

Hybrids between *P. ramosa* and *P. balduinii* have been suggested before (Smith & Ward 1976). Taxonomically, these have been called *P. balduinii* var. *chlorogena* Torr. & A. Gray. A single hybrid between *P. lutea* and *P. rugelii* was likewise mentioned by Smith and Ward (1976). They concluded that the majority of the pollen produced from this hybrid was nonfunctional.

It is also possible to discern direction of pollen flow based on the maternal inheritance of plastids and the plastid DNA haplotypes of the putative hybrids. Rare paternal or biparental inheritance is the rule in known gymnosperms (Reboud & Zeyl 1994; Wang et al. 2001) and some other plant groups may have varied parental inheritance of plastids (Zuccarello et al. 1999; Hansen et al. 2007). Plastids are known to be paternally inherited at very low frequency in *Arabidopsis thaliana* (L.) Heynh. (Azhagiri & Maliga 2007). However, plastids are generally inherited through maternal lineages (Reboud & Zeyl 1994). Because of this, when two individuals undergo sexual reproduction, it is the ovule-bearing individual that generally donates plastids to the offspring. With strict or primarily maternal inheritance, plastid haplotypes of different species in an interspecific cross can be used to determine direction of pollen transfer, without directly observing pollination events, assuming that there is consistent variation between the species. The *trn*L-F data presented here (Fig. 2) come from the plastid genome. Because the parents in both examples show distinct haplotypes for this DNA locus, the data can be used to indicate which species is the maternal donor in these putative hybrids. Both species pairs of hybrids show bidirectional hybridization (i.e., either species can be pollen donor).

Based on the low count of viable pollen (Smith & Ward 1976) and the rarity of these hybrids in the landscape, it seems likely that they are predominately sterile F1s. However, one population of *P. balduinii* × *P. ramosa* (*Abbott 14973* and *Abbott 14977*), at the western end of Santa Rosa Island in Escambia County, Florida, was observed to have more hybrid individuals than parental individuals, often not growing within close proximity of either parent. This suggests that in this population the hybrid may be self-sustaining and/or introgressing with the parents, and perhaps, merit taxonomic recognition.

Given that the putative hybrids have plastid haplotypes that match either of the parent species and that they have combinations of nrDNA that are polymorphic at identical variable sites to those same parental haplotypes, we infer that these individuals are indeed interspecific hybrids and not the product of other processes that may result in polymorphic DNA (e.g., incomplete lineage sorting). Ultimately, more study is needed to verify ploidy levels and pollinators in order to better understand these hybrid *Polygala*.

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