On biodiversity and conservation of the *Iris hexagona* complex (*Phaeiris*, Iridaceae)

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**Abstract.** Taxonomic revisions using newly available molecular data can have profound consequences for identifying areas of high endemism and, therefore, high conservation priority. A good example of the connection between taxonomy, biodiversity ecology, and conservation issues is genus *Phaeiris* (*Iris* subsect. *Hexagonae*), an endemic taxon of the southeastern United States and in particular *P. hexagona* (*I. hexagona*) (Blue Flag), perhaps the best-known species of this genus. Some authors recently provided evidence for the need to revise the taxonomy of the Blue Flag, which has usually been considered to consist of a single species, *P. hexagona*. Using molecular and bioclimatic analyses of Blue Flags from Florida and Louisiana, collected at their *loci classici*, we challenge the notion that *P. hexagona* is a single species, referring to the almost forgotten taxonomic context of *Phaeiris* as established by John K. Small and Edward J. Alexander nearly 90 yr ago. Our vision of the *P. hexagona* complex reinforces the current treatment of Southern Coastal Plain as a biodiversity hotspot. Our results also argue for the complex nature of endemism on the Florida Peninsula, and elsewhere on the Southern Coastal Plain, including a new interpretation of the well-studied Louisiana irises. We also propose that the valleys of Florida rivers and lakes are likely to harbor additional hidden biodiversity. Accurate taxonomy proved to be a heuristic tool to characterize better the actual level of biodiversity within Southern Coastal Plain as well as to describe the different areas endemism. The newly re-documented taxonomic diversity in broadly defined *P. hexagona* should also be considered in future conservation efforts.

**Key words:** bioclimatic analysis; Florida Peninsula; Louisiana irises; molecular phylogeny.

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The watersheds of different rivers commonly harbor flags of a single species or endemic plants. Small (1927:11).

**INTRODUCTION**

Modern phylogenetic methods, especially molecular approaches, have made it possible to identify many cryptic, unrecognized, or simply forgotten taxonomic entities (e.g., Mavrodiev et al. 2012a,b,c, 2013, 2014, 2015a, Yurtseva et al. 2016a,b, among others), some of which are potentially in need of conservation attention. Such new analyses have also provided new information on the prevalence of previously unrecognized areas.
of endemism (Mavrodiev et al. 2012a,b) as well as on the importance of environmental variables that may be fully congruent with the observed biodiversity (Mavrodiev et al. 2015b). In this context, the challenging of traditional taxonomic circumscriptions suspected to contain hidden taxa can also be an important tool for both ecologists and conservation biologists, especially when linked with actual or potential biodiversity hot spots (e.g., Mavrodiev et al. 2012a,b, Sokoloff et al. 2019, see also Liu et al. 2019).

A good example of such a connection between taxonomy, ecology, and conservation issues is the genus Phaeiris (I. subsect. Hexagonae; Appendix S1), an endemic taxon of the southeastern United States with its center of diversity in the Coastal Plain, particularly in the Mississippi River Delta region (e.g., Small and Alexander 1931, Crespo et al. 2015). Phaeiris has a high potential to contain cryptic or unrecognized species (e.g., Small and Alexander 1931) in need of conservation attention (Small 1931) and thus may also provide insights in identifying areas of endemism and environmental conditions that are congruent to the actual biodiversity of the genus.

Phaeiris hexagona (Iris hexagona) (Blue Flag; Small 1925a) is perhaps the best-known species of the genus. The major goal of this study is to evaluate critically circumscription of Phaeiris hexagona in the context of the growing recognition that:

1. the Southern Coastal Plain of North America (SCP) is a biodiversity hot spot (Noss et al. 2015), and
2. the Florida peninsula, in particular, is an area of high endemism (reviewed in James 1961 and Reece and Noss 2014).

Therefore, we have to discuss:

1. the possible cryptic diversity of the Florida Blue Flag, which is impossible without the general taxonomic outlook on the Louisiana irises;
2. the possible structure of the SCP biodiversity, in particular the possibility of river valleys to be potential sources of cryptic biodiversity;
3. the future of the conservation status of the Phaeiris hexagona complex, as well as the rest of the genus Phaeiris.

To achieve these goals, we perform bioclimatic and cladistic analyses of all extant non-hybrid taxa of the genus Phaeiris, with special attention on those related to P. hexagona.

At least five related taxa have been segregated from Phaeiris hexagona (Iris hexagona) in the past (e.g., Small 1930a, Small and Alexander 1931, Foster 1937), namely Phaeiris albispiritus (I. albispiritus; Small 1929a), P. giganticaerulea (I. giganticaerulea; Small 1929b), P. rivularis nom. prov. (I. rivularis; Small 1927), P. kimballiae nom. prov. (I. kimballiae; Small 1925c), and P. savannarum (I. savannarum; Small 1925b; see Table 1 and Appendix S1–S3 for details and complete names).

Today, however, a common belief is that this group consists of only one (e.g., Hamlin et al. 2017) to three species (e.g., Meerow et al. 2011; Table 1). Here we challenge that idea using molecular sequence data in a bioclimatic context thus referring to the almost forgotten taxonomic assessment of Phaeiris done long ago by John K. Small and Edward J. Alexander (Small and Alexander 1931), who described numerous species of Phaeiris, mostly from the Mississippi River.

Table 1. Summary of the taxonomic treatments of Phaeiris hexagona and non-extinct related species referred to in this study.

| Small and Alexander (1931) | Viosca (1935); Foster (1937); Hamlin et al. (2017) | Henderson (2002); Meerow et al. (2007, 2011, 2017) | This study |
|---------------------------|--------------------------------------------------|-----------------------------------------------|-------------|
| Iris giganticaerulea      | Iris hexagona                                    | Iris. giganticaerulea                         | Phaeiris giganticaerulea |
| I. hexagona               | I. hexagona                                      | I. hexagona                                   | P. hexagona |
| I. kimballiae             | I. hexagona                                      | I. savannarum                                 | P. kimballiae |
| I. rivularis              | ignored                                          | I. savannarum                                 | P. rivularis |
| I. savannarum             | I. hexagona                                      | I. savannarum                                 | P. savannarum |
The natural hybrids between synonym of and I. giganticaerulea (1931) soon were criticized by the whose primary scienti
United States (Small 1903, see also James 1961 ago in the which was well established more than a century
of the enormous et al. (2015). It not congruent with earlier visions considered part of a biodiversity hotspot by Noss (2017), vieso (1935, also see Hamlin et al. 2017). In short, Viosca argued that only three species from Iris subsect. Hexagonae occur in southeastern Louisiana: Iris foliosa (today conceptualized as a synonym of I. brevicaulis; Henderson 2002), I. fulva, and I. giganticaerulea (Viosca 1935), the actual synonym of I. hexagona (Viosca 1935; Table 1). The natural hybrids between I. fulva and I. hexagona (i.e., I. giganticaerulea), as well as their introgressants, form a spectrum of variations that Viosca (1935) mistakenly equated with many species recognized by Small and Alexander (1931).

The taxonomy of Viosca (1935) and Foster (1937) established the framework for most subsequent biosystematic interpretations of the Louisiana irises (e.g., Anderson 1949, 1961, Riley 1938, Foster 1937, also see Hamlin et al. 2017). In short, Viosca argued that only three species from Iris subsect. Hexagonae occur in southeastern Louisiana: Iris foliosa (today conceptualized as a synonym of I. brevicaulis; Henderson 2002), I. fulva, and I. giganticaerulea (Viosca 1935), the actual synonym of I. hexagona (Viosca 1935; Table 1). The natural hybrids between I. fulva and I. hexagona (i.e., I. giganticaerulea), as well as their introgressants, form a spectrum of variations that Viosca (1935) mistakenly equated with many species recognized by Small and Alexander (1931).

The taxonomy of Viosca (1935) and Foster (1937) established the framework for most subsequent biosystematic interpretations of the Louisiana irises (e.g., Anderson 1949, 1961, Grant 1981, Arnold 2000, 2004, Arnold et al. 2004, 2010, 2012, Hamlin et al. 2017, Sung et al. 2018, among others). As a result, their actual diversity has been unrecognized until the new analyses presented in this paper, which largely confirm Small’s original observations.

Indeed, Hamlin et al. (2017:148), based on limited sampling, reduced the entire phylogeny of Iris subsect. Hexagonae to the three-taxan statement with Iris brevicaulis and I. fulva forming a clade regarded as the sister of I. hexagona. It is remarkable that Hamlin et al. (2017:148) cited Viosca (1935) as the major taxonomic source for their study.

Such solution contradicts the results of many current biological investigations of the SCP, considered part of a biodiversity hotspot by Noss et al. (2015). It not congruent with earlier visions of the enormous floristic diversity of the SCP, which was well established more than a century ago in the first edition of Flora of the southeastern United States (Small 1903, see also James 1961 and Austin et al. 1987). In this context, the high diversity of iridaceous plants (Small 1925a, 1927, 1929a, b, 1930a, Small and Alexander 1931) would be just another case of a widespread phenomenon.

The taxonomic interpretation of the blue flags of Louisiana and Florida indeed has been partly challenged by Randolf (1966) and especially by Henderson (2002), Ward (2008, 2011), and Meerow et al. (2007, 2011, 2017). For example, the studies of Hamlin and Arnold (2014, 2015) that combined the populations of three taxa (iris giganticaerulea, I. hexagona, and I. savannarum) under I. hexagona “... may be more a scenario of species divergence than population level adaptation” (Meerow et al. 2017:318, see also Meerow et al. 2011).

In this study, we continue the molecular investigation of the non-hybrid species of Phaeiris in Florida and Louisiana. We evaluate our results in a bioclimatic framework thus referring to the general framework of the fundamental ecological niche (Hutchinson 1957, reviewed in Colwell and Rangel 2009 and Holt 2009). After clarifying the species-level systematics of Phaeiris hexagona, we then will place our findings in the broader contexts of the Louisiana irises and the SCP’s overall biodiversity, its possible structure, and conservation significance. Specifically, we predicted that the revised circumscription of this genus will be in theory consistent with areas of endemism already identified in the Florida peninsula.

**Materials and Methods**

**Taxonomic framework**

Genus Phaeiris is currently accepted in circumscription of 13 species (Crespo et al. 2015; Appendix S1). Following others (Small 1929a, Small and Alexander 1931, see also Henderson 1994, 2002), we a priori accepted that among these 13 taxa, Phaeiris albispiritus, P. brevicaulis, P. fulva, P. giganticaerulea, P. hexagona, P. kimballiae nom. provis., P. rivularis nom. provis., and P. savannarum are the only species with a presumably non-hybrid origin (Table 1; Appendix S1–S3). We also a priori accepted P. brevicaulis (iris foliosa) and P. fulva in their traditional circumscriptions (e.g., Henderson 1994, Hamlin et al. 2017), although more work is needed to estimate the relationships and composition of these
binomials more rigorously (Small and Alexander 1931, see also Trahan 2007:4, 10).

**Field sampling**

Important to our research is the concept of the *locus classicus* (type locality), or location from which the holotype of a particular taxon was collected. Appendix S1 contains the details of the estimation of the investigated locations of *Phaeiris giganticaelulea* (our tissue number 3790), *P. hexagona* (our collections 3758, 3759, and 3760), *P. kimballiae* (our collection 3779), *P. rivularis* (our collection 3772), and *P. savannarum* (our collection 3773). The goal was to sample all of these taxa exactly in its loci classici, or at least in the actual trustable localities (Figs 1–2).

We regard *Phaeiris albispiritus* (the Ghost Iris of Small, 1929a) as an extinct Floridian endemic that we cannot sample (Appendix S1), even if the albino forms of *P. savannarum* have been reported from the vicinity of the Caloosahatchee River and Fort Myers (Meerow et al. 2011, 2017).

**Climatic data analyses**

The type localities of *Phaeiris rivularis*, *P. kimballiae*, and *P. savannarum* (Fig. 1), as well as the Florida locations of *P. hexagona* (Fig. 1), lie in the Level IV Ecoregions of Florida (Omernik 1987, Griffith et al. 1994, Omernik and Griffith 2014), published online by National Health and Environmental Effects Research Lab, US Environmental Protection Agency Office of Research and Development1. The ecoregions of type localities are (Fig. 2): level IV Coastal Marsh of the Southern Coastal Plain (SCP) for *Phaeiris rivularis*, level IV Floodplains/Low Terraces (SCP) for *P. kimballiae*, and level IV Eastern Florida Flatwoods (SCP) for *P. savannarum*.

Finally, the Florida locations of *P. hexagona* occupy the level IV Gulf Coast Flatwoods (SCP; Fig. 2).

To evaluate the climatic variability associated with each presumed non-hybrid taxon of *Phaeiris*, we first downloaded all records in iDigBio and GBIF and extracted for each locality the

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*Fig. 1. Localities of the five Blue Flags studied in this paper. Loci classici (type localities) of *Phaeiris giganticaerulea, P. kimballiae, P. rivularis, P. savannarum*, and sampled trustable Floridian locations of *Phaeiris hexagona*, as estimated by Henderson (2002), Ward (2011), and Meerow et al. (2011). See Appendix S1 for more detail.*
19 environmental variables available in world-clim$^2$ at 1-km$^2$ resolution. To reduce collinearity and describe each of the lineages’ climatic envelope using few variables, we performed a principal component analysis (PCA) after scaling the environmental variables by subtracting from each value the mean and dividing by the standard deviation of the dataset.

Next, using the first two principal components we performed a search to find the optimal number of clusters present in the data using the gap statistic based on the k-means algorithm with 1000 bootstrap replicates (Tibshirani et al. 2001). Finally, we run a UPGMA hierarchical clustering based on the climate of the type localities in order to test their climatic similarities. The cluster was performed using the Euclidean distance among the values of the first two PC.

After describing the climate envelope of all known localities, we asked if the climate envelope...
of the type localities in Florida and Louisiana have differences that could suggest that the populations are subject to different environments. We constructed 10-km² radius buffers around the coordinates of the type localities and extracted the values of the 19 worldclim environmental layers at a 1-km² resolution at every pixel inside each buffer. Next, we repeated the PCA and evaluated the optimal number of clusters using the gap statistic based on the k-means algorithm with 1000 bootstrap replicates. All analyses were done in R 3.5.2, and the gap statistic was calculated in the cluster package in R (Maechler et al. 2012).

DNA markers, extraction, amplification, cloning, and sequencing

In their analysis of a large dataset, Li et al. (2011) confirmed the earlier proposal of Kress et al. (2005) that the internal transcribed spacer (nrDNA ITS-1 and 2 loci, ITS hereinafter, see Baldwin et al. 1995) should be incorporated into the core barcode for seed plants, with ITS showing more discriminatory power than the three other popular plant barcodes (the chloroplast-based rbcL and matK, and the trnH-psbA intergeneric spacer or IGS). Wheeler and Wilson (2014) demonstrated the high utility of the ITS region for the molecular systematics of Limniris (Iris subgenus Limniris), a close relative of Phaeiris (Mavrodiev et al. 2014, Crespo et al. 2015). Therefore, we regard the ITS sequence data as a powerful tool for molecular systematics of plants in general (also see Ren et al. 2019) and Iridaceae in particular (Wheeler and Wilson 2014).

Methods of DNA extraction, as well as amplification, cloning, and sequencing of the ITS region, follow Mavrodiev et al. (2012c). The region was amplified and sequenced using primers: ITS_LEU1 (GTCCACTGAACCTTATCATTTAG; Bruce Baldwin in Ballard et al. 1998), and our originally designed PhaeirisITSF (GATATGCT-TATTAACCGGGTA).

Cladistic analyses

As an out-group of Phaeiris, we used the sequence data of Limniris virginica (L.) Rodion. (see Mavrodiev et al. 2014 for more detail).

Because of the high number of Intra-Individual Site Polymorphisms (2ISPs) within the ITS sequences, the standard molecular alignments of the eight ITS clones of Phaeiris giganticaerulea, P. hexagona (three accessions: 3758, 3759, and 3760), P. kimballiae, P. rivularis, and P. savannarum were represented as their Union Ambiguity Consensuses using the SeaView version 4.0 (Gouy et al. 2010) and analyzed as described in Potts et al. (2014).

The seven consensuses of the ITS sequences were aligned with the direct ITS sequences of Phaeiris brevicaulis, P. fulva, and Limniris virginica by MUSCLE (Edgar 2004), as implemented by SeaView version 4.0 (Gouy et al. 2010) and analyzed using cost step-matrices (Potts et al. 2014). The conventional Fitch parsimony (reviewed in Kitching et al. 1998) was also performed with the data specified as a standard (with the symbols ACTGMRWSYKVHDB).

A three-taxon statement analysis (3TA, reviewed in Kitching et al. 1998) of the same matrix using Wagner parsimony (see Kitching et al. 1998 for the review) was conducted as described in Mavrodiev and Madorsky (2012) with all of the three-taxon statements (3TSs) weighted uniformly.

The Williams-Siebert (WS) representation of the multistate molecular alignment as a 3TS matrix (Williams and Siebert 2000, as implemented in Mavrodiev and Madorsky 2012) was performed in TAXODIUM version 1.2 (Mavrodiev and Madorsky 2012) following the command: taxodium inputfilename.csv -ium -ob -og -nex with an operational out-group defined as Limniris virginica.

All maximum parsimony (MP) analyses (including bootstrap resampling) were done in PAUP* version 4.0 (Swofford 2002) as described in Mavrodiev and Madorsky (2012).

RESULTS

Sequencing and cloning

Due to the extremely high number of 2ISPs on the sequences of the ITS region (Table 2), we were not able to generate any direct sequences of Phaeiris giganticaerulea, P. hexagona (three accessions), P. kimballiae, P. rivularis, and P. savannarum. However, we were able to obtain eight ITS clones for each of these taxa. In contrast, for P. fulva and Limniris virginica we were able to obtain the ITS sequences without cloning. Lacking living material of P. brevicaulis, this taxon was represented by a single ITS sequence obtained

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from the GenBank (see above). Thus, the total number of ITS sequences in our analyses was 59 (56 ITS clones plus three direct sequences), from which 58 sequences were obtained in this study.

**Cladistic analyses**

The total number of characters in the final ITS alignment was 713, consisting of 266 constant characters (proportion = 0.373), 272 parsimony-informative characters, and 175 variable characters that were parsimony-uninformative. The 3TS-WS representation of the same multistate alignment contained 4986 binary characters (3TSs), all parsimony-informative.

The maximum parsimony (MP) analysis of the same matrix using standard Fitch parsimony yielded a single, well-supported most parsimonious tree (length = 898, CI = 0.7817, RI = 0.5692; Fig. 3).

The MP analysis of the ITS dataset using the step matrix to define additional steps for changes between monomorphic and polymorphic sites (Potts et al. 2014) also yielded a single well-supported most parsimonious tree (length = 1090, CI = 0.7229, RI = 0.4933; Fig. 3).

The MP analysis of the 3TS-WS binary representation of the multistate molecular alignment again yielded a single well-resolved most parsimonious tree (length = 5653, RI = 0.8662; Fig. 3).

All three MP trees are topologically identical, with *Phaeiris hexagona* being monophyletic, and *P. savannarum*, as treated by Henderson (2002; i.e., incl. *P. kimballiae* and *P. rivularis*) clearly non-monophyletic (Fig. 3, Table 1).

We also found that *Phaeiris giganticaerulea* is not the part of the *P. hexagona* clade, nor did it form a clade with *P. kimballiae*, *P. rivularis*, or *P. savannarum* (Fig. 3).

In other words, the key result of our cladistic analyses is the monophyly of *P. hexagona*, as this taxon has accepted in the *Flora of North America* (Henderson 2002; Table 1) and paraphyly of *P. savannarum*, as it defined in the same floristic treatment (Henderson 2002; Fig. 3, Table 1).

*Phaeiris brevicaulis* and *P. fulva* do not form a clade, unlike in the analysis of Hamlin et al. (2017), instead, the former is a well-supported sister to the rest of the genus (Fig. 3).

*Phaeiris* itself is monophyletic and well supported, as in Mavrodiev et al. (2014; Fig. 3).

**Climatic data analyses**

The first two principal components (PC) of the environmental variables at the type localities and the localities obtained from iDigBio and GBIF explain 75% of the variance. The first PC pertains mainly to temperature and the second PC to precipitation variables (Appendix S4: Tables S1 and S2). The optimal number of clusters found by the gap statistics was five (Fig. 4A, Appendix S4: Fig. S1). Cluster analysis of climate at the type localities showed a cluster formed by *Phaeiris hexagona* and *P. rivularis*, another formed by *P. kimballiae* and *P. giganticaerulea*, and *P. savannarum* on its own (Fig. 4B).

Similarly, the first two PC from the environment immediately around the type localities explained 81% of the variance. The first PC was related mainly to temperature and the second PC mainly to precipitation. The exceptions were that variables bio12, bio14, bio17, and bio19 (precipitation) were mainly related to the first PC, whereas bio2,
Fig. 3. The results of three different cladistic analyses of the molecular sequence data of all of the presumable non-hybrid taxa of the genus *Phaeiris*. All three trees (A–C) are topologically identical and therefore established as a single figure. The support values from different analyses (A–C) are written in the slashes below branches. (A) Single most parsimonious tree from the MP analysis of the unordered multistate matrix of ITS sequence data of all of the presumable non-hybrid taxa of the genus *Phaeiris*. Matrix has established and designed as in Potts et al. (2014). (B) Single most parsimonious tree from the standard MP analysis (Fitch parsimony) of the same multistate data matrix (A). (C) Nested most parsimonious hierarchy of patterns from the MP analysis (Wagner parsimony) of the Williams-Siebert 3TSs representation of the same matrix (A). The values of this out-group were fixed as the values of *Limniris virginica* (3TA). The bootstrap values from different analyses (A–C) are written in the slashes below branches.
bio5, and bio7 (temperature) were more related to the second PC (Appendix S4: Tables S1 and S2).

Cluster analysis identified four clusters as the optimal number with *P. kimballiae* and *P. rivularis* having almost identical climate (Fig. 4C). In short, our bioclimatic analysis showed that the type localities of *Phaeiris giganticaerulea*, *P. hexagona*, *P. kimballiae*, and *P. savannarum* are clearly different in their climate and environment (Figs 4, 5), and these differences are generally congruent with our analyses of the molecular data (Figs 3, 6).

**DISCUSSION**

Our results strongly suggest high levels of cryptic diversity within *Phaeiris* and that this diversity is consistent with climatic and geographic (riverine) gradients and biogeographic centers of endemism that may be of even higher conservation priority than previously recognized. The strong congruence between taxonomy and the results of bioclimatic analyses is also argued in favor of the concept of the fundamental ecological niche (Hutchinson 1957, reviewed in Colwell and Rangel 2009 and Holt 2009), which seems relevant to current and future investigations of the biodiversity of the SCP in general and Floridian Peninsula in particular. In the remainder of the discussion, we explore the evidence for the cryptic diversity of *Phaeiris*, discuss the possible reasons why such diversity was overlooked...
in spite of the original description, which has proven to be remarkably accurate, and then conclude with an examination of the factors that likely led to the differentiation of the cryptic species within this complex and their potentially dire conservation status.

The general taxonomic outlook on the Louisiana irises is pre-submitted as an Appendix S2. But as it is perhaps clear from what is above, the implications of our results are not purely taxonomic. We believe that accurate taxonomy proved to be a heuristic tool to characterize better the actual level of biodiversity within the SCP, to describe the areas endemism (such as Floridian peninsula or the Delta of Mississippi), and help to establish the future conservation priorities within these areas of the SCP.

On cryptic diversity of the Florida Blue Flags

_Phaeiris giganticaerulea_, _P. hexagona_, _P. kimballiae_, _P. rivularis_, and _P. savannarum_ are morphologically distinctive (e.g., Small and Alexander 1931) and ecologically well-defined entities (Figs. 2, 4–6; Appendix S4: Fig. S1). Their external differences (Figs. 7–9, Appendix S3: Figs. S1–S5) are also intuitively clear (Mavrodiev and Yurtseva 2017).

At the same time, the ITS region of these taxa is highly polymorphic (Table 2). The reasons for this polymorphism are unclear and may differ among taxa; however, we tend to agree with Potts et al. (2014) that the treatment of 2ISPs as analytically informative characters seems to be relevant despite not knowing what underlies the observed polymorphisms.

Our results are also congruent with those of Meerow et al. (2007, 2011, 2017) in showing the high genetic dissimilarity among the distinct populations of _Phaeiris_, even when using different methods and molecular markers (Meerow et al. 2007, 2011, 2017). While most apomictic and asexual (clonal) plants have considerable genetic variation (reviewed in Bengtsson 2009), we do not see much evidence for this phenomenon in the non-hybrid species of _Phaeiris_. In general, the plants of non-hybrid species of _Phaeiris_ are clearly not asexual, even if asexual reproduction is common within some hybrids of _Phaeiris_ (e.g., Burke et al. 2000) and already Small (1930b) and Viosca (1935) have mentioned some issues with the seed reproduction of the Floridian and Louisianan blue flags. In general, apomixis is not common in Iridaceae, reported as far as we know only for _Belamcanda chinensis_ (Yu et al. 2017).

We also would like to stress that the UA consensuses of the ITS sequences are not simply different from each other (Table 2), but have differences that are analytically informative. For example, the results of our cladistic analyses show that _Phaeiris hexagona_ forms a strongly supported clade (Fig. 3) and also supports species-level recognition of _P. giganticaerulea_, _P. hexagona_, _P. kimballiae_, _P. rivularis_, and _P. savannarum_ (Fig. 3), as collected at their type localities (Figs 1, 2).

Some individuals identified as _Phaeiris hexagona_ in herbaria collections are from localities that are bioclimatically more similar to _P. giganticaerulea_ and _P. savannarum_ than to many other _P. hexagona_ individuals (Fig. 4A). On the other hand, bioclimatic analyses from only the

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**Fig. 5.** Results of the bioclimatic analyses (2). Principal component analysis showing the climatic distribution around trustable Floridian locations of _Phaeiris hexagona_ as estimated by Henderson (2002), Ward (2011), and Meerow et al. (2011) and the type localities (loci classic) of _Phaeiris giganticaerulea_, _P. kimballiae_, _P. rivularis_, and _P. savannarum_, based on the first and third principal components. See Materials and Methods and Appendix S4 for more detail.
five type localities suggest that their environmental conditions are significantly different (Figs. 4C, 5).

Although mostly focused on population genetics, Meerow et al. (2011, 2017) also argued for taxonomic separation of *Phaeiris savannarum* from *P. hexagona*. They also found that most of the studied populations of the Floridian blue flags are genetically dissimilar, including recognition of *P. giganticaerulea* and a restricted concept for *P. hexagona*, agreeing with our results (Figs. 3–5). Although the genetically peculiar and relatively homogenous populations from the Big Cypress Swamp, Lake Wales Ridge, and Polk Uplands all were assigned to *P. savannarum* by Meerow et al. (2011), they may deserve recognition at the specific rank, especially if the Caloosahatchee population of the same taxon (located close to the type locality of *P. savannarum*) also is genetically distinct (e.g., Meerow et al. 2011). Moreover, the Highlands and Coastal plants of *P. savannarum* are phenotypically distinct (Meerow et al. 2017). This issue calls for more research. Thus, the taxonomic diversity of *Phaeiris* may actually exceed what we currently understand.

**Florida Blue Flags and river valleys as potential sources of cryptic biodiversity**

Patterns of biological diversity on any peninsula may have numerous causes (Battisti 2014). The biodiversity of Florida as well as North American Coastal Plain were reviewed in Noss et al. (2015) and Allen et al. (2019), the latter a bioinformatics study guided by spatial phylogenetics. Most such studies assume that the biotic richness is well known, so the task is to relate that known richness to the available phylogenetic and spatial information to estimate patterns of phylogenetic diversity.

In describing *Iris kimballiae*, Small (1925c:59–60) noted that this taxon “…has been found in the swamps along the lower Apalachicola River and in swamps in the vicinity of the lagoons in the coastal region of both sides of the upper part of the peninsula.” As summarized by Ward (2011:235), Small originally (1925c) reported [*Iris Kimballiae*] from both Apalachicola and northeast Florida, but later restricted it to the western station, the northeastern plants becoming his *Iris rivularis*. Thus, eventually Small limited the distribution range of *I. kimballiae* to “swamps, often calcareous” and “coastal regions of Apalachicola delta” (Small and Alexander 1931:352). Small also found an undescribed flag in extensive colonies along tributaries of the St. Marys River that he named *Iris rivularis* (Small 1927). Thus, the type localities of *Iris kimballiae* and *I. rivularis* are in two different river basins. In the same manner, the type specimens of *I. savannarum* were collected on the southern side of the Caloosahatchee River, Florida (Small 1925b) and the type of *I. giganticaerulea* was collected near Morgan City, Louisiana (Small 1929b), on the
banks of Atchafalaya River, which is part of the Mississippi River Delta.

Therefore, from the five presumably non-hybrid taxa of *Phaeiris* related to *P. hexagona*, only the Floridian *P. hexagona* cannot be clearly linked with the basin of a single river. All population clusters of *Phaeiris* reported in Meerow et al. (2011) can be easily and clearly linked to basins of certain Florida rivers and/or lakes. For example, his Big Cypress Swamp sample occurs only in the Turner River basin, his Polk Uplands sample only in the Lake Manatee lower watershed, and his North-Central Gulf Costal sample in the Waccasassa and Lower Suwanee rivers. Other populations are associated with Caloosahatchee River, Upper Myakka River valley (SB), Withlacoocchee River valley (WH), and so on. Thus, the diversity of *Phaeiris* seems to be structured not only within Florida’s ecoregions (Fig. 2) but also through the basins of rivers and/or lakes.

Small (1927:11) explicitly stated that “the watersheds of different rivers commonly harbor flags of a single species or endemic plants.” In other words, just as the Apalachicola River sustains the unique *Phaeiris kimbaliae* and the Caloosahatchee River harbors *P. savannarum*, many other rivers in Florida (and westward to Louisiana) may also support unique blue flags that are genetically distinct at the species level.

Shortly after Small (1927), the Russian botanist A. D. Fursaev (1937) also noted that the floodplain of the Volga River (the largest river in European Russia) sustains many cryptic endemic taxa (reviewed in Mavrodiev et al. 2012). As Fursaev also pointed out, this endemism (at least partially) is tied to the different micro-evolutionary processes taking place in the river’s floodplain, which rapidly adapt the valley’s populations of flowering plants to long periods of extensive flooding and eventually results in rapid speciation, a proposition that was named Fursaev’s Theorem (Mavrodiev et al. 2012). Even if such cryptic biodiversity may have different causes (Mavrodiev et al. 2012), this theorem in principle could apply to taxa immediately associated with any large river (Fursaev 1937).

That the valleys of rivers commonly harbor the endemic plants, as put forth by Small (1927) and Fursaev (1937), eventually led us to formulate the River Valley Hypothesis of the biodiversity of the Floridian Peninsula and SCP in general. Building on this concept, we note that the rivers and their floodplains on the Florida as well as SCP are centers of endemism not only for blue flags or other flowering plants, but also for many other types of organisms in Florida such as freshwater fishes (Robins et al. 2018). In freshwater clams, for example, 17 of the 59 native Florida species (29%) are confined to a single river basin (Williams et al. 2014, their Table 5.2). Certain aquatic and semi-aquatic salamanders and  

Fig. 7. Flower of *Phaeiris rivularis*. Flower of *Phaeiris rivularis*, as currently photographed in its *locus classicus* by the first author (the upper part of image) and as drawn by Mary Emily Eaton (Mitich 2000) in Small (1927; the lower part of image). See Appendix S3: Fig. S2 for more detail.
snakes in Florida also have distributions restricted to just several rivers in spite of seemingly suitable habitat in between (Ashton and Ashton 1988). Even in more purely terrestrial organisms such as pocket gophers, the Apalachicola River forms a major barrier to gene flow (Chambers et al. 2009).

In the discussed context, it is also worth to mention the Wallace’s (1854) riverine barrier hypothesis, that seems to be relevant for the wide spectrum of living organisms, including the flowering plants (e.g., Nazareno et al. 2017, 2019). In short, it is likely that many new discoveries are waiting for scientists who investigate potential endemism of the North American Coastal Plain (Noss et al. 2015).

**Closing note: on the conservation future of the genus Phaeiris**

If the number of species of *Phaeiris* is actually large, then almost every population of *Phaeiris* may be represented by the unique genotype (e.g., Meerow et al. 2011) or even by described or undescribed (cryptic) endemic taxa. If saving as many taxa of *Phaeiris* as possible is a conservation goal, then the entire genus should be the target of urgent conservation. In other words, within our taxonomic framework (Appendix S1, S2) and that of Small and Alexander 1931), nearly every population of *Phaeiris* adds to its genetic diversity and should be considered for conservation.

A very different vision of iris conservation was put forth by Viosca (1935:54, italics and bold ours, see also Anderson 1936:507): “I have shown that man-made factors such as ditching, deforestation, and cattle grazing under certain conditions, not only increase parental stocks, but encourage natural hybridization. If the *innumerable forms exhibited by the irises of this region represent* distinct species . . . then of course most of them are doomed, for any unique plant is soon removed by some garden enthusiast and if not of hardy stock may soon perish. *If, on the other hand, all but the four wide spread species recognized herein are natural hybrids, then of course there is nothing to prevent new hybrid seedlings from developing annually in places where the parental stocks of fulva and giganticaerulea are still numerous.* My observations strongly favor this latter view. If this view is correct, as the *scant* but rather conclusive experimental evidence also tends to prove, then *these irises have a bright rather than a gloomy future . . . .*”

These suggestions have not withstood the test of time. Viosca’s bright future for Louisiana irises was directly challenged by founder of the Society for Louisiana Irises (SLI) Marie Caillet (Caillet et al. 2000), by Trahan (2007), as well as by Greater New Orleans Iris Society, that currently established the special SLI and GNOIS Launch
Major Projects (2015) to conserve Louisiana Iris species.

For example, Caillet et al. (2000:35) suggested: “... Unfortunately, the species (of *I. subsect. Hexagonae*) continue to grow scarcer in native habitats. Natural interspecific hybrids are even rarer. This scarcity can be attributed to human intrusion through expansion of cities and development of once wild areas. Drainage of natural wet areas is the foremost problem. Water is essential for growth and distribution of Louisiana irises. The species *Iris nelsonii* is most endangered because of its very restricted habitat. Native stands are nearly absent now and saving *I. nelsonii* in its habitat will be difficult. *Iris fulva* is also in danger and natural populations have decreased drastically over the past 70 yr. ... Fortunately, Louisiana iris species continue to thrive in personal and public display gardens. Distribution of plants or seeds to many areas where these irises are not now grown is an important conservation practice.”

Based on his almost 10 yr of field observations, Trahan (2007) was even more skeptical. He believed that the future of the Louisiana iris species is dim and thus the whole complex is on a death march because of residential development expanding into the many natural iris habitats and “... the once large masses of iris vanished. Additionally, the oil industry excavated canals from the Gulf of Mexico through coastal roadways to oil-related business locales in an effort to gain shorter and quicker routes to supply offshore drilling. These canals dissected swamps and marshes, allowing salt water from the Gulf to enter into iris habitat, destroying the irises in those areas. Swamps were and are being drained to create land more suitable for agriculture. Aircraft spraying herbicides very often overspray the intended fields, killing the irises growing adjacent to them... Development of roadways and highways also destroys iris habitat. In an effort to save time and money, herbicides are used to control the growth of weeds along roadway ditches. It seems as though the decision has been made that it is easier and more cost effective to poison grass once every three months rather than cut the grass once each month, and the iris generally do not survive the poisonous solutions” (Trahan 2007:7–9).

Trahan (2007) summarized that “… there are too many reasons why the naturally growing Louisiana iris species will be extinct in the near future” and predict “that the first to disappear from the wild will be *Iris nelsonii*, followed by *I. brevicaulis*, *I. giganticaerulea*, *I. hexagona*, and finally, *I. fulva*” (Trahan 2007:9).

The observations of Caillet et al. (2000), Trahan (2007) and others build on those of Small (1931:177), who as one of the founders of the defunct Louisiana Iris Conservation Society seriously doubted the bright future of Louisiana
irises already in 1931, writing “… we confidently calculate that through rural improvements and urban growth about eighty per cent of the iris fields of a half century ago have been ravaged or destroyed!”

The conservation situation with the Phaeiris hexagona complex in Florida may also be dire. We already mentioned that the type locality of the Ghost Iris (Phaeiris albispiritus) is likely destroyed (Appendix S1) and that we were not able to find rare P. brevicaulis in Florida (Appendix S1) despite several intensive field trips. Our careful searches in the Apalachicola and St. Marys river basins showed that both P. kimballiae and P. rivularis are now also extremely rare, both species should be considered as endangered, and protected before it is too late.

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Notes

1ftp://newftp.epa.gov/EPADataCommons/ORD/Ecoregions/fl/fl_eco_pg.pdf
2http://www.worldclim.org/bioclim

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**Supporting Information**

Additional Supporting Information may be found online at: http://onlinelibrary.wiley.com/doi/10.1002/ecs2.3331/full