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Leaf Phenology of *Danaea geniculata* (Marattiaceae) in a Submontane Tropical Forest, Brazil

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Abstract.—Ferns adapt their phenological characteristics such as leaf production, leaf fertility and leaf mortality to the habitat conditions optimizing their opportunities to grow, reproduce, and disperse. The leaf phenology of the herbaceous, dimorphic fern *Danaea geniculata* was studied in a Brazilian submontane tropical forest and compared with several other fern species worldwide. Plants of *D. geniculata* held an average of 8.11 ± 2.16 leaves that were produced and died at similar rates of 4.44 ± 2.16 leaves y^{-1} and 4.20 ± 2.28 leaves y^{-1}, respectively. Leaf lifespan was 24.7 ± 7.5 mo for sterile leaves and 5.6 ± 0.6 mo for fertile leaves. Leaf production and fertility increased with rainfall, but decreased with temperature, because of the local climate, which is characterized by higher rainfall during the colder winter months. On the other hand, leaf mortality increased during drier and hotter months. Leaf production and fertility of other species were fit into three categories and compared with *D. geniculata*. The leaf phenology of *D. geniculata* neither coincided with species at the same site nor with species within the same family (Marattiaceae), indicating that even coexisting or closely related species can adapt individually to the macro- and microclimatic parameters of their habitats.

Key Words.—ferns, fertility, leaf dimorphism, plant phenology, seasonality

The climate on Earth varies with the seasons in annual cycles but also undergoes acyclic long-term changes. Phenology is the study of the occurrence and periodicity of biological events (e.g., flowering) in response to the cyclic changes of biotic and environmental variables (Lieth, 1974; Rathcke and Lacey, 1985). Currently, the study of phenology is extended to investigate the effect of climate change on the ecology of organisms, communities, and ecosystems, as well as species interactions, food chains, and food webs (Visser, 2016). Consequently, phenological studies are fundamental to our understanding of the ecological responses of plants to the periodically changing environment and to the life cycles of coexisting organisms (e.g., herbivorous insects) as well

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as to global climate change, and are of utmost importance for cultivation of crop species as well as the conservation of native species.

Because of their lack of flowers, fruits and seeds, phenological studies of ferns have focused on leaves and spore production. Published data come mainly from Brazil, Mexico, Puerto Rico, and Taiwan (e.g., Sharpe, 1997; Mehltreter and Palacios-Rios, 2003; Lee, Lin, and Chiou, 2009; Farias *et al.*, 2015) with a strong bias on tree ferns (e.g., Chiou, Lin, and Wang, 2001; Schmitt, Schneider, and Windisch, 2009; Mehltreter and García-Franco, 2008; Neumann, Schneider, and Schmitt, 2014). In most tropical ferns that have been monitored, at least some of the phenological variables such as leaf production, leaf growth, fertility, or leaf mortality were seasonal and consequently correlated with rainfall and/or temperature (Mehltreter, 2008), photoperiod (Müller *et al.*, 2015) or even light quality (Arens and Sánchez Baracaldo, 2000; Arens, 2001). Former studies could not provide clear phenological patterns for groups of species within the same habitat or the same species across a larger area of distribution because of the relatively few studied fern species. Two exceptions are Lee, Lin, and Chiou (2009) who showed that phenologies of 16 species may vary significantly within the same habitat, and the mangrove fern *Acrostichum danaeifolium* Langsd. & Fisch., which has been studied independently at two different sites (Mexico and Puerto Rico) with a similar intraspecific phenological variation (Mehltreter and Palacios-Rios, 2003; Sharpe, 2010). Consequently, we aimed in our study to (1) determine the phenological characteristics (production, lifespan, fertility, growth, and mortality of leaves) of the dimorphic fern species *Danaea geniculata* Raddi, (2) examine the effect of rainfall and temperature on the observed phenological patterns of *D. geniculata*, and (3) compare our results with phenological studies of other fern species worldwide.

Materials and Methods

Study area and climate.—The study was conducted in the *Mata do Estado* (600 ha), a Brazilian submontane tropical forest of Pernambuco (07°36′55″ S, 35°30′44.5″ W; 600–640 m a.s.l.). This area displays a floristic richness of approximately 400 species of angiosperms and 90 species of ferns (Lucena, 2009). The forest canopy of 25–30 m height is dominated by species such as *Dialium guianense* (Aubl.) Sandwith, *Eschweilera ovata* (Cambess.) Miers, *Helicostylis tomentosa* (Poepp. & Endl.) Rusby, *Pouteria bangii* (Rusby) T.D. Penn., *Sloanea guianensis* (Aubl.) Benth., *Virola gardneri* (A.DC.) Warb. and *Vochysia thyrsoidea* Pohl (Ferraz and Rodal, 2006).

According to Peel, Finlayson, and MacMahon (2007), the climate of the study area is classified as hot and wet (Aw). Data from the nearest meteorological station (São Vicente Férrer) at 4.5 km from the study area (07°35′16″ S, 35°29′20.5″ W), provide evidence for two well-defined seasons (data available from Agência Pernambucana de Água e Clima, APAC, 2017). The wet season lasts from January to September and the dry season, defined by less than 50 mm of monthly precipitation (Worbes, 1995), lasts from October to

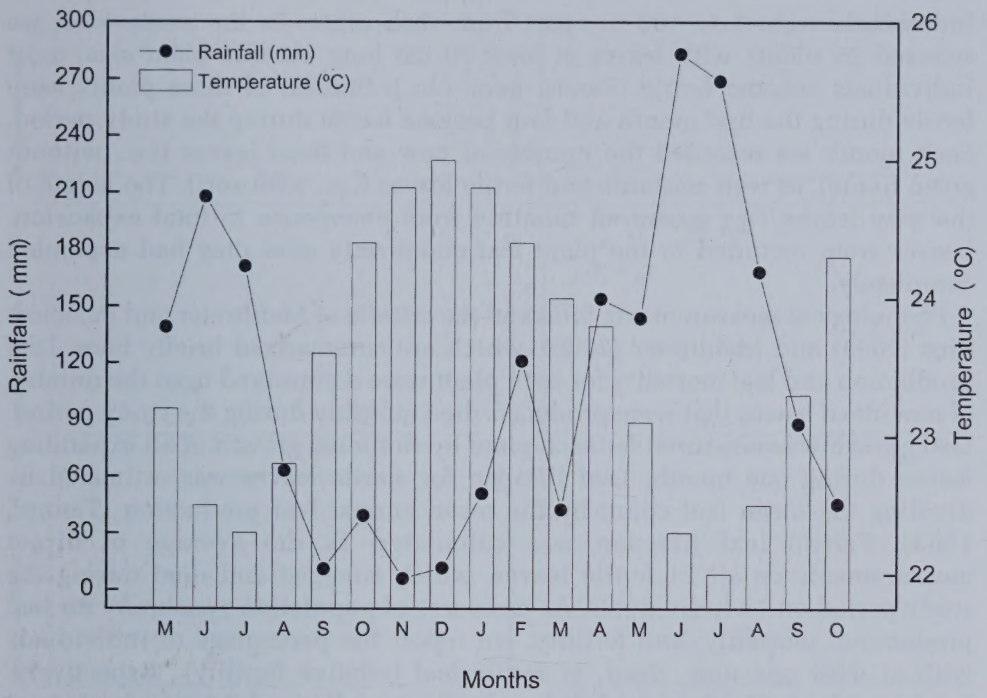

FIG. 1. Rainfall and temperature data for the study period from May 2012 to October 2013 at São Vicente Férrer, Pernambuco, Brazil (APAC, 2017).

December. The mean annual precipitation is 1103 mm and the mean annual temperature is 24°C (data for 2000-2016; APAC, 2017). During the study period, annual precipitation deviated strongly from the mean (2012: 830 mm, 26 % lower; 2013: 1430 mm; 28% higher than average annual rainfall). During the driest month of the study period in November 2012, rainfall was 25% below the monthly mean and during the wettest month in June 2013, precipitation was 68% above the monthly mean. Mean monthly temperatures ranged from 21.7°C during the wet season in July 2013 to 25.0°C during the dry season in December 2012 (Fig. 1; APAC 2017).

Study species.—*Danaea geniculata* (Marattiaceae) is an herbaceous terrestrial fern with dimorphic leaves. Fertile leaves are much narrower, ca. 25% longer, and more short-lived than sterile leaves according to measurements of plants in our study. This species is characterized by an erect rhizome with roots emerging on all sides, leaves arranged in rosettes, petioles with 1–2 nodes and terminal pinnae never replaced by buds. Its geographical distribution ranges from the Greater Antilles, the island of Guadeloupe and Mexico to Bolivia and Brazil (Christenhusz 2007, 2010).

Data collection.—Phenological data were collected between May 2012 and October 2013 (18 mo), although fertility was monitored until November 2013. All individuals were growing within an approximate area of 1 ha in the forest understory along a wet ravine and thus experienced similar habitat conditions.

Individuals were 3 to 100 m apart from each other. In the study area, we selected 25 plants with leaves at least 70 cm long. At this plant size, most individuals become fertile (Farias, pers. obs.). Sixteen of these plants were fertile during the first month and four became fertile during the study period. Each month we recorded the number of new and dead leaves (i.e., without green tissue), as well as sterile and fertile leaves (i.e., with sori). The length of the new leaves was measured monthly from emergence to total expansion. Leaves were included in the plant leaf count only after they had expanded completely.

Phenological measurements followed the criteria of Mehltreter and Palacios-Rios (2003) and Mehltreter (2006), which are summarized briefly here. Leaf production and leaf mortality for each plant were determined from the number of new/dead leaves that were produced/died monthly during the study period. Leaf growth was measured for each plant by summing growth of all expanding leaves during one month. Leaf lifespan for sterile leaves was estimated by dividing the mean leaf count by the mean annual leaf production (Tanner, 1983). Fertile leaf lifespan was calculated as the average of direct measurements on all 21 fertile leaves, which emerged and died during the study period on 14 individuals. As measures of population synchrony for leaf production, mortality, and fertility, we report the percentage of individuals with at least one new, dead, or fertile leaf (relative fertility), respectively. Leaves with sori were considered as fertile regardless of the developmental stage of their sporangia.

Data analyses.—Spearman correlation tests (Zar, 1999) were performed to analyse the relationship between monthly data of phenological variables (leaf production, fertility, leaf growth and leaf mortality) and climatic variables (temperature and rainfall). In addition, we correlated the population synchrony of each phenological variable with climate data, and plant size (= length of largest leaf) with the number of sterile and fertile leaves (Spearman). We performed a parametric paired t-test to detect significant differences of phenological variables (leaf production, leaf growth, leaf mortality) between seasons (dry season of 3 months and wet season of 9 months), and between the monthly production of sterile and fertile leaves. For fertility, a non-parametric Wilcoxon signed-rank test was used to detect differences between seasons. For the latter analyses, data for replicated months were averaged. Statistical analyses were performed with R 3.2.5 software (R Core Team, 2016).

Results

Plant leaf count, leaf production and leaf lifespan.—Plant leaf count for *D. geniculata* (Fig. 2A, $n = 25$) averaged 8.11 ± 2.16 leaves of which 5.5% were fertile (Fig. 2B; Table 1). Plants produced about four leaves per year (0.37 ± 0.18 leaves mo^{-1}; Table 1). Although sterile leaf production occurred during most months, it peaked twice per year. The first peak occurred during the wet season in June and August 2012 and June 2013 (> 0.6 new sterile leaves per plant and $> 64\%$ leaf producing individuals). The second peak occurred

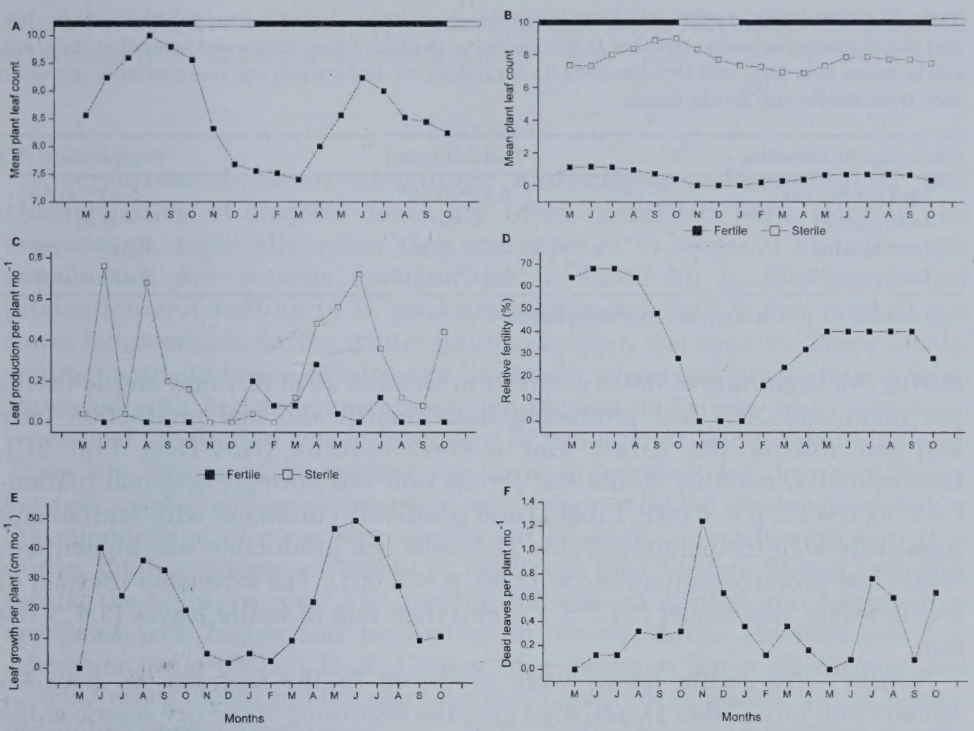

FIG. 2. Phenological data for *D. geniculata* from May 2012 to October 2013 in a Submontane Tropical Forest in Brazil. A – Total leaf count per plant, B – Sterile and fertile leaf count per plant, C – Leaf production per plant, D – Relative fertility (% of fertile individuals), E – Leaf growth per plant, and F – Number of dead leaves per plant. Bar on top: black = rainy season, white = dry season.

TABLE 1. Monthly means of phenological variables of *D. geniculata* (N = 25) per year and season. The dry season lasts 3 months. Means ± SD. Letters indicate significant differences between seasons ($p < 0.05$).

	Annual mean	Wet season	Dry season
Plant leaf count	8.11 ± 2.16	8.08 ± 2.19 [a]	8.20 ± 2.16 [a]
Fertile	0.45 ± 0.33	0.56 ± 0.41 [a]	0.12 ± 0.14 [b]
Sterile	7.66 ± 1.92	7.51 ± 1.88 [a]	8.08 ± 2.12 [b]
Leaf length (cm)	80.61 ± 12.25	80.07 ± 13.11 [a]	80.95 ± 12.96 [a]
Fertile	85.04 ± 6.26	83.80 ± 6.12 [a]	87.21 ± 9.31 [a]
Sterile	80.73 ± 12.91	80.07 ± 12.87 [a]	80.95 ± 12.95 [a]
Leaf production	0.37 ± 0.18	0.46 ± 0.23 [a]	0.10 ± 0.12 [b]
Fertile	0.06 ± 0.09	0.08 ± 0.13 [a]	0 [b]
Sterile	0.31 ± 0.11	0.37 ± 0.14 [a]	0.10 ± 0.12 [b]
Leaf growth	19.80 ± 6.55	24.02 ± 8.57 [a]	7.15 ± 4.28 [b]
Leaf mortality	0.35 ± 0.19	0.23 ± 0.17 [a]	0.70 ± 0.47 [b]

TABLE 2. Correlation coefficients (r) of monthly phenological variables (population means, left, and population synchrony, right) of *D. geniculata* with mean temperature and rainfall at the study site between May 2012 and October 2013. Leaf production, leaf growth and leaf mortality represent data from sterile and fertile leaves.

Phenological variables	Rainfall (mm)	Temperature (°C)
Leaf production	0.59*/0.58*	-0.57*/-0.59*
Leaf growth	0.69*	0.82**
Leaf fertility	0.53*/0.48*	-0.84**/-0.81**
Leaf mortality	-0.51*/-0.51*	0.46ns/0.45ns

* $p < 0.05$; ** $p < 0.001$; *ns* – not significant.

during the beginning of the dry season in October 2013 (0.4 new sterile leaves per plant and 36% leaf producing individuals), and leaf production only stopped during the driest and hottest months (Nov-Dec; Fig. 2C). Consequently, monthly sterile leaf production was strongly seasonal (paired-$t = 7.50$, $n = 25$, $p < 0.001$; Table 1) and positively correlated with rainfall and negatively with temperature (Table 2). Sterile leaf production was higher than fertile leaf production (paired-$t = 12.99$, $p < 0.001$). The estimated lifespan of sterile leaves was longer (24.7 ± 7.5 mo) than that of fertile leaves (5.6 ± 0.6 mo).

Fertility.—All fertile leaves were produced in the wet season, between January and July (Table 1), and died after the beginning of the dry season at the latest in October (Fig. 2D). Consequently, fertile leaf count was significantly higher during the wet season (signed-rank $W = 66$, $p < 0.005$; Table 1). The fertile leaf count in 2012 (1.24 leaves plant^{-1}) was nearly twice as much as in 2013 (0.64 leaves plant^{-1}). The mean production of fertile leaves and relative fertility of plants (% of fertile individuals) were significantly correlated with increasing rainfall and decreasing temperatures (Table 2). The maximum relative fertility of the population was higher in 2012 (17 plants, 68%) than in 2013 (10 plants, 40%). Seven plants (28%) produced fertile leaves in both wet seasons, ten plants only in 2012 and three plants only in 2013. Consequently, 80% of the plants were fertile at least once during the study period. Leaf length was positively correlated with the number of sterile ($r = 0.48$, $p < 0.05$) and fertile leaves ($r = 0.67$, $p < 0.001$).

Leaf growth.—Mean leaf growth was 19.80 ± 6.55 cm plant^{-1} mo^{-1} (Table 1), but differed significantly between seasons (paired-$t = 8.94$, $p < 0.001$; Table 1), increasing with rainfall and decreasing with temperature (Table 2). It reached its maximum of 44.76 ± 28.44 cm in June 2013 (wet season) and its minimum of 1.70 ± 3.23 cm in December 2012 (dry season, Fig. 2E).

Leaf mortality.—About one out of three leaves died each month (0.35 ± 0.19 mo^{-1}) but leaf mortality increased seasonally (paired-$t = 5.19$, $p < 0.001$; Table 1) with rainfall but not with colder temperature (Table 2). Whereas sterile leaves died mainly between November (dry season) and March (wet season), fertile leaves died earlier between August (wet season) and November (Fig. 2F). The percentage of individuals with dead leaves was negatively correlated with

rainfall (Table 2) and reached its maximum (56%) in November 2012 and its minimum (0%) in May 2013 (wet season).

Discussion

Correlations between phenology and climate.—Phenological leaf characteristics of tropical ferns are often correlated with rainfall and temperature, especially when they are exposed to seasonal climates with pronounced dry seasons (summarized in Table 3). In this study, leaf production and fertility of *D. geniculata* increased with higher rainfall and lower temperatures during winter (June to August, wet season). These results contrast with studies in Mexico and SE-Brazil, where leaf production of ferns increased with higher temperatures, and studies in Taiwan and Costa Rica, where leaf production was independent of temperature and rainfall (Table 3). There are two possible reasons for these differences: (a) the relationship between rainfall and temperatures at each site, and (b) the water availability at the microhabitat of some fern species. At all Mexican study sites rain falls during the hot summer, whereas in NE-Brazil (São Vicente Férrer) rain falls during the colder winter. The parallel increase of rainfall and temperature correlates with higher leaf production in the deciduous Mexican tree fern *Alsophila firma* (Baker) D. S. Conant of a montane forest (Mehltreter and García-Franco, 2008) and the climbing fern *Lygodium venustum* Sw. of a semi-deciduous coastal forest (Mehltreter, 2006), both of which are exposed to a dry winter season of six months (Table 3). On the other hand, the mangrove fern *Acrostichum danaeifolium* (Mehltreter and Palacios-Rios, 2003) and the montane forest fern *Marattia laxa* Kunze (Flores-Galván, 2015) produce their leaves independent from rainfall (Table 3), because water availability at their respective habitats, the mangroves and the mountain ravines, apparently does not limit their growth during the dry season. At the Taiwanese and Costa Rican sites without a dry season, the leaf production is independent of the abundant rainfall ($>$ 4000 mm year^{-1}), but still dependent on the larger annual temperature changes in Fushan (Taiwan) than the small temperature changes in La Selva (Costa Rica) (Sharpe and Jernstedt, 1990; Chiou, Lin, and Wang, 2001; Lee, Lin, and Chiou, 2009). In some fern species, however, leaf production occurs independently from rainfall and temperature. The striking differences between the two species at São Vicente Férrer (Brazil) *Danaea geniculata* and *Didymochlaena truncatula* (Sw.) J. Sm., with the former species phenologically dependent on rainfall and temperature and the latter species independent of both climate parameters (Table 3), might be the consequence of their differing microhabitats. Future phenological studies should include the measurement of microhabitat conditions close to each plant individual to understand the possible impact of microhabitat characteristics on their leaf phenology.

In summary, leaf production in tropical ferns is highly variable and can be classified into at least three categories with a subcategorization: species (1) dependent on increasing rainfall and (a) increasing or (b) decreasing

TABLE 3. Correlations between climate and leaf characteristics of ferns from different sites. [1]Mehltreter and Palacios-Rios (2003), [2]Mehltreter (2006), [3]Mehltreter and García-Franco (2008), [4]Flores-Galván (2015), [5]Sharpe and Jernstedt (1990), [6]this study, [7]Farias *et al.* (2015), [8]Schmitt and Windisch (2012), [9]Schmitt, Schneider, and Windisch (2009), [10]Chiou, Lin, and Wang (2001), [11]Lee, Lin, and Chiou (2009). Correlation: + significantly positive, − significantly negative, 0 none, ? unknown. * average of sterile and fertile leaves.

Country	Site	Alt. (m)	Annual rainfall (mm)	Min-Mean-Max Temp. (°C)	Dry season (mo)	Species	Leaf dimorphism	Leaf life span (mo)	Leaf production		Leaf fertility		Leaf mortality	
									Rain-fall	Temp.	Rain-fall	Temp.	Rain-fall	Temp.
Mexico	La Mancha	0	1198	22-25-29	6	*Acrostichum danaeifolium*[1]	yes	7.7	0	+	+	+	?	?
						Lygodium venustum[2]	weak	5.6*	+	+	?	?	−	+
	Huatusco	1300	1950	18-20-23	4-6	*Alsophila firma*[3]	no	10.0*	+	+	?	?	−	+
	Coatepec	1600	1350	16-19-22	1-4	*Marattia laxa*[4]	no	20.5	0	+	?	?	−	+*
Costa Rica	La Selva	100	>4000	25-26-27	0	*Danaea wendlandii*[5]	yes	39.0	?	?	+*	?	?	?
Brazil	S. Vic. Férrer	600	1155	21-23-25	3	*Danaea geniculata*[6]	yes	21.1	+	−	+	−	−	+
						Didymochlaena truncatula[7]	no	12.0*	0	0	0	0	−	0
	Novo Hamburgo	20	1500	15-19-25	0	*Cyathea atrovirens*[8]	no	?	0	+	0	+	0	0
	Morro Reuter	570	1700	14-18-23	0	*Dicksonia sellowiana*[9]	no	?	0	0	0	+	0	0
Taiwan	Yangmingshan	560	>4000	14-19-25	1	*Cibotium taiwanense*[10]	no	20.0*	0	0	?	?	?	?
	Fushan	600	>4000	12-18-24	0	16 spp.[11]	some spp.	4-30	0	+	+	+	−	0

temperatures and species (2) independent from rainfall only but dependent on increasing temperatures or (3) independent from both rainfall and temperature. Leaf fertility is similarly variable among species and can be classified in the same three categories (1a, 1b, 2, 3). Most species belong to the same categories of leaf production and leaf fertility, with the exception of some species in which leaf fertility is depending more on rainfall than leaf production (e.g. *A. danaeifolium*; switch from category 2 to 1a; Table 3) and *Dicksonia sellowiana* Hook. in which leaf fertility is depending on increasing temperature while leaf production is not (switch from 3 to 2; Table 3). Leaf mortality is less variable than leaf production and fertility. In most species of seasonal climates leaf mortality increases during the dryer and hotter season, whereas in most species of aseasonal climates leaf mortality is independent from rain and temperatures (Table 3).

Seasonal fertility of dimorphic species.—In our study, *D. geniculata* was seasonally fertile, produced fewer fertile than sterile leaves, and the former were shorter-lived than the sterile leaves. The same combination of a few, seasonally produced, and short-lived fertile leaves has been reported for other dimorphic species (Sharpe and Jernstedt, 1990; Mehltreter and Palacios-Rios, 2003; Watkins, Churchill, and Holbrook, 2016). Fertile leaves of dimorphic species have a strongly reduced leaf area (Wagner and Wagner, 1977), and act as strong carbon sources because of lower carbon fixation than respiration rates (Chiou *et al.*, 2005; Britton and Watkins, 2016; Watkins, Churchill, and Holbrook, 2016). The significant carbon costs of fertile leaves in dimorphic species might be the reason for its seasonal production and short-lived existence. First, the high carbon costs may allow dimorphic plants only a limited production of fertile leaves and only during the most resource-rich, wet season, when sufficient sterile leaves provide the necessary carbon balance. Second, maintenance costs possibly limit fertile leaf lifespan to a few months, just enough time until spores have been released. Currently, there is little evidence that drought stress is responsible for fertile leaf mortality during the dry season, because fertile leaves of the dimorphic *Lomariopsis vestita* have similar xylem specific conductivity and higher (more positive) water potentials, and are less exposed to cavitation than sterile leaves (Watkins, Churchill, and Holbrook, 2016). Consequently, dimorphic ferns might not be able to keep their fertile leaves during the dry season, because of the combination of increasing respiratory rates of fertile leaves and decreasing carbon fixation rates of sterile leaves due to limited water availability.

Relative fertility.—The relative fertility and fertile leaf count of *D. geniculata* varied considerably between the wet seasons of 2012 and 2013. Annual changes of fertility have been rarely reported for ferns. For instance, *A. danaeifolium* had a plant fertility of 33% in two consecutive years, with a turnover of 10% of fertile plants becoming sterile the following year and 10% sterile plants turning fertile the second year (Mehltreter and Palacios-Rios, 2003). These results suggest that the current relative fertility of a fern population cannot be easily predicted for subsequent years. Especially in dimorphic ferns with a high production cost of fertile leaves, reproductive

phenology may be mostly affected by the net carbon balance of previous seasons and years according to physiological experiments performed by Watkins, Churchill, and Holbrook (2016). The net carbon balance might also affect the susceptibility of plant individuals to environmental triggers (water, photoperiod, and temperature). In addition to environmental factors, internal factors involved in floral induction of angiosperms are plant developmental age and gibberellic acid concentrations (Song, Ito, and Imaizumi, 2013). All these factors should be further investigated in ferns to understand the similarities and differences between the floral induction of angiosperms and fertile leaf induction in ferns.

Interspecific phenological variation.—Fern species from the same site may vary considerably in their phenologies because of species-specific traits or differences in their microhabitats, which may be the case for *Danaea geniculata* (present study) and *Didymochlaena truncatula* (Farias *et al.*, 2015). Even if both species occur in the same area, the former species grows along the river bank and the latter on the mountain slopes (Farias, pers. obs). Consequently, *D. geniculata* might be able to keep its sterile leaves even during the dry season because of its presumably wetter microhabitat.

On the other hand, even closely related species that share similar leaf traits (e.g., leaf dimorphism) may adapt phenologically to changing environmental conditions when they grow in different areas. For example, *D. geniculata* from Brazil and *D. wendlandii* Rchb.f. from Costa Rica (Sharpe and Jernstedt, 1990) share leaf dimorphism with long-lived sterile and short-lived fertile leaves. However, they did not classify into the same leaf production categories (see above and Table 3), and *D. geniculata* had about two times shorter sterile leaf lifespan than *D. wendlandii,* possibly because of the lower annual rainfall and the more pronounced dry season at our study site (Table 3). Another member of the family Marattiaceae, *Marattia laxa*, has long-lived monomorphic leaves, and classified into another leaf production category than both *Danaea* species (Table 3). A comparison of four tree fern species, two of each of families Cyatheaceae and Dicksoniaceae (Table 3) also shows that each species follows its own phenological pattern. An extreme example of closely related species that differ in their leaf phenology are the two tree ferns *Alsophila firma* and *Cyathea bicrenata* Liebm., which can coexist even in the same forests but occupy slightly different microhabitats. The former species is deciduous and grows typically in the shady understory, whereas the latter is evergreen and can thrive even in the full sun (Mehltreter and Garcia-Franco, 2008). In conclusion, leaf phenological patterns may differ considerably among species that live together within the same habitat and among species within the same family (e.g., Marattiaceae), when they adapt phenologically their leaf production and fertility to the specific climate conditions of their microhabitats. Future research should study the phenology of single species over a broad geographic range to understand the variability and adaptability of phenological traits to changing climatic conditions. Controlled studies in climate chambers could also provide more insights into the correlations

between specific environmental variables (photoperiod, light quality, rainfall and temperature, soil) and phenological responses of ferns.

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Mating System and Genetic Variability of the Endangered Endemic Aquatic Lycophyte, *Isoëtes yunguiensis*, in China Determined using AFLP Markers

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Abstract.—In this study, mating system, genetic diversity, and genetic structure of the endangered endemic aquatic lycophyte *Isoëtes yunguiensis* in China was investigated using AFLP markers. The estimates of outcrossing rates (t_m = 1.200) indicate that diploid *I. yunguiensis* is a predominantly outcrossing species. Six selected AFLP primer pairs used in the study amplified 195 reproducible bands, 160 of which were polymorphic (PPB = 82.05%), indicating significant genetic diversity at the species level. AMOVA revealed that 40.12% of the genetic variation was attributable to differences between populations and the rest (59.88%) to variability within populations. High outcrossing rates **and t**he historical accumulation of genetic variation may be responsible for the high levels of genetic diversity observed in the *I. yunguiensis* population. To maintain the current level of genetic diversity for this species, we recommend increasing *in situ* conservation sites.

Key Words.—*Isoëtes yunguiensis*, mating system, genetic variability, conservation management, AFLP

Studies of mating system, genetic diversity, and population structure, as well as the spatial distribution of genotypes within populations of rare and endangered plant species are necessary to establish effective and efficient

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conservation management strategies for endangered species (Pometti *et al.*, 2013).

Isoëtes yunguiensis Wang Q.F. & W.C Taylor (Isoëtaceae; Wang *et al.*, 2002) found in the Yunnan-Guizhou Plateau in southwest China are distinct from the more widespread *I. japonica* A. Br. based on spore morphology (microspores of *I. yunguiensis* are smaller than those of *I. japonica*) and chromosome number (*I. yunguiensis* is diploid with a chromosome number of 2n = 22, whereas those of *I. japonica* can be 2n = 66, 67, 77, 87, 88, or 89). *Isoëtes yunguiensis* is perennial, distributed in ponds, riverside meadows, and marshes at elevations of 1200-2200 m in Yunnan and Guizhou Provinces, China. In recent decades, the number and size of *I. yunguiensis* populations have declined rapidly due to degeneration of primary habitats by human activity. Field investigation showed that five *I. yunguiensis* populations (designated as KM1-4, XD1), in Heilongtan, Songhuaba, Xiaoshao, Shuangshao, and Xuandian, Kunming City, Yunnan Province based on specimen records, are extinct (Pang *et al.*, 2003) (Table 1). In our recent fieldwork, two populations, in Pingba County and Hongfenghu Lake, Guizhou Province, were discovered in China. The species is now endangered and listed among the first category of the Key Protected Wild Plants in China (Yu, 1999). Chen *et al.* (2007) reported genetic variation of 46 individuals from the population remaining at Pingba (Guizhou Province, China) using random amplified polymorphic DNA (RAPD) fingerprinting. However, little is known about the mating system and genetic diversity of *I. yunguiensis*, even though a thorough understanding of them may be a prerequisite for conserving the species.

AFLPs have been used to evaluate genetic diversity and outcrossing rates in plant species, including seven endangered *Isoëtes* species (*I. taiwanensis* De Wol, *I. asiatica* (Mak.) Holub, *I. jejuensis* H.K. Choi, C.H. Kim, & J. Jung, *I. hallasanensis* H.K. Choi, C.H. Kim, & J. Jung, *I. coreana* Y.H. Chung & H.K.Choi, *I. japonica*, and *I. hypsophila* Hand.-Mazz.) from East Asia (Kim, Shin, and Choi, 2009; Chen *et al.*, 2010a) and ferns (e.g., *Blechnum spicant* (L.) Sm and *Dryopteris affinis* ssp. *affinis* (Lowe) Fraser-Jenk.; Peredo *et al.*, 2013).

The principal aims of this study were to estimate the outcrossing rates and genetic diversity in natural populations of *I. yunguiensis* in China using AFLP markers, thereby providing genetic information of remnant wild populations for future *in situ* and *ex situ* conservation and management programs.

Materials and Methods

Sample collection and DNA extraction.—From 2003 to 2015, the historic geographic distributions of *I. yunguiensis* in China were investigated. The two extant populations (designated as PB and QHH) were found in Guizhou Province in China during our field surveys (Fig. 1). Both populations were small, each containing fewer than 50 individuals (Table 1). At the sampling sites, elevation, latitude, and longitude were recorded using a Global Positioning System (GPS), and the habitat characteristics of *I. yunguiensis* were recorded. Because the species is now endangered in China and the two

TABLE 1. Geographic distribution, location, habitat, and sample size of *Isoëtes yunguiensis* populations studied

Population code	Extant/ extinct population	Locality	Latitude/ longitude (N/E)	Altitude (m)	Habitats	Population size	Population area (m^2)	Sample size	Vouchers/ references
PB	Extant	Pingba, Guizhou	26°25′/106°17′	1365	Valley swamp	40-50	50-60	19	HCAS 75043/the present study
QHH	Extant	Hongfenghu, Guizhou	26°29′/106°24′	1247	Valley swamp	40-45	45-50	18	The present study
KM1	Extinct	Heilongtan, Kunming City,Yunnan	25°02′/102°42′	2000	Pond				KUN 0002883/ Pang *et al.*, 2003
KM2	Extinct	Songhuaba, Kunming City, Yunnan	25°02′/102°42′	2000	Reservoir				KUN 0002885/ Pang *et al.*, 2003
KM3	Extinct	Xiaoshao, Kunming City, Yunnan	25°02′/102°42′	2000	Rice-field stream				KUN 0002888/ Pang *et al.*, 2003
KM4	Extinct	Shuangshao, Kunming City, Yunnan	25°02′/102°42′	2160	Rice-field stream				KUN 0002886/ Pang *et al.*, 2003
XD1	Extinct	Xuandian, Kunming City, Yunnan	25°56′/103°25′	2080	Rice-field stream				KUN 65680/ Pang *et al.*, 2003

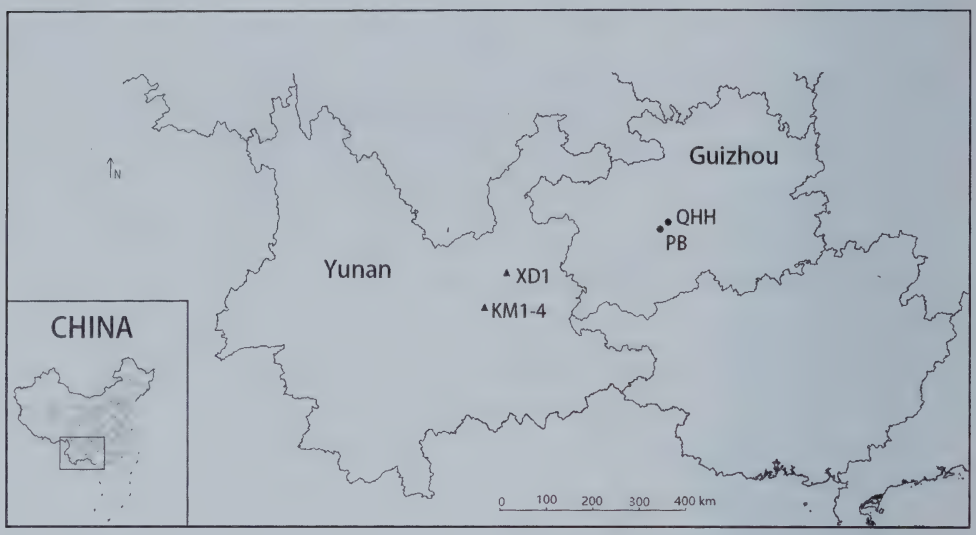

FIG. 1. Distribution map of *Isoëtes yunguiensis* populations sampled in the present study. • Sites of extant populations. ▲ Sites of extinct populations (see Pang *et al.*, 2003). Codes correspond to populations in Table 1.

extant populations are small, in order to minimize human influence on these populations, only 18 and 19 samples were collected from each population. A total of 37 individuals from the two remaining populations of *I. yunguiensis* in this study were collected. Individuals in each study population were sampled at a minimum distance of 1 m from one another. Details on collection sites, population size, and sampling size are listed in Table 1. Approximately 5-10g of young leaves were harvested from each plant and immediately dried in a sealed Ziplock plastic bag containing about 50g of silica gel.

Total DNA was extracted from 0.3 to 0.5g of silica-dried leaf tissue using the CTAB method (Doyle and Doyle, 1987).

AFLP-PCR amplification.—AFLP analysis was performed using a procedure modified from Vos *et al.* (1995). 2μL (50ng/μL) of total genomic DNA was digested using 3 units of EcoRI and MseI endonuclease mixture (New England Biolabs, Beijing, China) in a total volume of 20μL for 3 h at 37°C. Then, 10μL of ligation solution containing 5pM EcoRI adaptor, 50pM MseI adaptor (Table 2), and 5 units of T4 DNA ligase were added to the digests in a total volume of 20μL. The ligation mixture was incubated overnight at 22°C in a thermocycler.

PCR pre-amplification was performed in a 20μL solution containing 2.5μL 10×Taq buffer, 0.5μL dNTPs, 1μL primer EcoR I, 1μL primer MseI (Table 2), 0.5μL Taq polymerase (TianGen, Beijing, China) and 4μL template DNA for the following the thermal profile: initial melting at 94°C for 3 min, followed by 25 cycles of 94°C for 30s, 56°C for 1 min and 72°C for 1 min.

For selective PCR amplification, six primer combinations (EcoRI+3/MseI+3) (Table 2) were chosen among 64 sets that were screened for variability, and the 15-fold diluted pre-amplification product was used as template. An aliquot of

TABLE 2. DNA sequences of AFLP primers, adaptors from each primer combinations of *Isoëtes yunguiensis* in two populations studied

Adaptor	Sequence
EcoRI adaptor	5′-CTCGTAGACTGCGTACC-3′
	3′-CATCTGACGCATGGTTAA-5′
MseI adaptor	5′-GACGATGAGTCCTGAG-3′
	3′-TACTCAGGACTCAT-5′
EcoRI + 0/MseI + 0 primer	5′-GACTGCGTACCAATTC-3′ (E0)
	5′-GATGAGTCCTGAGTAA-3′ (M0)
EcoRI + 3/MseI + 3 primer	E2×M1 E+AAG/ M+C AA
	E2×M2 E+AAG/ M+C AA
	E5×M1 E+ACC/ M+C AA
	E6×M1 E+ACG/ M+C AA
	E6×M2 E+ACG/ M+C AA
	E7×M1 E+AGC/ M+C AA

4μL diluted pre-amplification DNA was added to 5.5μL of selective amplification mixture (1μL EcoRI+3 primer, 1μL MseI +3 primer, 0.5μL dNTPs, 2.5μL 10×Taq buffer [TianGen], 0.5μL Taq polymerase), and amplified with the thermal cycle profile: 94°C for 3 min, then 13 cycles of 94°C for 30s, 65°C (decreasing by 0.7°C) for 30s and 72°C for 1 min, followed by 23 cycles of 94°C for 30s, 56°C for 30s and 72°C for 1 min, then 72°C for 5 min.

The amplification products added with 7.5μL loading buffer were denatured at 94°C for 5 min and electrophoresed on a 6% denaturing polyacrylamide gel on a 50.5 × 35 cm DYCZ-20D DNA Sequencing System (Thermo). Silver staining was conducted as described by Bassam, Caetano-Anollés, and Gresshoff (1991). Sizes of selective amplification products were estimated using a 50 bp DNA ladder (TianGen, Beijing, China). The AFLP analyses were repeated three times.

Data analyses.—MATING SYSTEM.—AFLP markers are typically dominant. Only intense and unambiguous bands (100-500 bp) were manually scored as presence (1) or absence (0), and the data matrix of the AFLP phenotypes was constructed for further analysis. The program MLTR 3.4 (Ritland, 2009) is based on the multilocus mixed-mating model and the estimation procedure of Ritland and Jain (1981), which assumes that the progeny are derived from either random mating (outcrossing) or self-fertilization. Markers exhibiting dominance, such as AFLPs and RAPDs, can also be used in the multilocus mixed-mating model (Ritland, 2002). Dominant loci are allowed in MLTR (Ritland, 2002). Thus, using the software MLTR 3.4 (Ritland, 2009), we calculated the following mating system parameters: estimation of multilocus (the multilocus population outcrossing rate, t_m) and single locus outcrossing rates (the single locus population outcrossing rate, t_s), multilocus correlation of outcrossed paternity (rpm), single locus correlation of paternity (rps), correlation of t_m among progeny arrays (rt), and the fixation index of maternal parents (the single locus inbreeding coefficient of maternal parents, F). The biparental inbreeding rate (difference of outcrossing rate) was also estimated

TABLE 3. Mating system parameter of *Isoëtes yunguiensis* populations studied in China

Parameter	PB	QHH	Species level
t_m	1.200 (0.003)	1.200 (0.000)	1.200 (0.000)
t_s	0.973 (0.134)	0.909 (0.000)	0.880 (0.039)
t_m–t_s	0.227 (0.134)	0.291 (0.000)	0.320 (0.039)
rp(m)	0.198 (0.198)	0.252 (0.001)	0.279 (0.053)
rp(s)	-0.999 (0.054)	-0.374 (0.000)	0.031 (0.417)
rp(s)– rp(m)	-1.197 (0.206)	-0.626 (0.000)	-0.248 (0.375)
rt	-0.999 (0.014)	-0.999 (0.000)	-0.999 (0.000)
F	-0.200 (0.006)	-0.200 (0.001)	-0.133 (0.034)

t_m, Multilocus outcrossing rate; t_s, Single-locus outcrossing rate; t_m-t_s, Difference of outcrossing rate or biparental inbreeding; rp(m), The multilocus correlation of paternity; rp(s), The single-locus correlation of paternity; rp(s)–rp(m), Parents correlation; F, Inbreeding coefficient of the maternal parents; Numbers in parentheses are standard deviations (SD).

following Ritland (1990) as t_m - t_s. The standard errors for these parameters were calculated from 1,000 bootstraps with resampling of individuals within families.

GENETIC DIVERSITY AND GENETIC STRUCTURE.—To evaluate genetic diversity, the percentage of polymorphic bands (PPB), the Shannon index of diversity (*I*), and the gene diversity index (*H*) were calculated. At the species level, the coefficient of gene differentiation (G_{st}) and the level of gene flow (Nm) were measured. Nm among populations was indirectly estimated by the formula $Nm = 0.5(1 - G_{st})/ G_{st}$ (McDermott and McDonald, 1993). All calculations were estimated using POPGENE program Version 1.32 (Yeh, Yang, and Boyle, 2000). In order to describe genetic variability within and among populations, analysis of molecular variance (AMOVA) was performed using ARLEQUIN 3.5.2 (Excoffier and Lischer, 2015). The Nei and Li (1979) coefficient for measuring pairwise band similarities between individuals was calculated using NTSYSpc ver. 2.02 (Rohlf, 1998). The dendrogram (UPGMA) of all individuals was computed using the unweighted pair-group method with an arithmetic average using NTSYSpc ver. 2.02 (Rohlf, 1998). Significance tests were performed after 1000 permutations.

RESULTS AND DISCUSSION

Mating system analysis.—t_m and t_s were higher at the species level and in all populations (t_m= 1.200, $t_s \geq 0.880$, respectively), indicating that the *I. yunguiensis* populations studied are mostly outcrossing (Table 3). High outcrossing rates in *I. yunguiensis* based on AFLP markers in the study were similar to those reported in other studies. For example, Maki and Asada (1998) using allozyme data reported that homosporous pteridophyte *Polystichum otomasui* Sa. Kurata. predominantly favors outcrossing. Similarly, recent studies indicated that the European diploid rock fern *Asplenium fontanum* (L.) Bernh. subsp. *fontanum*, which has high migration capacity, reproduces preferably via outcrossing despite a low frequency of inbreeding based on

allozymic loci (Bystriakova *et al.*, 2014). A high degree of outcrossing was also reported in an endangered and endemic fern *Adiantum reniforme* var. *sinense* Y. X. Lin. in China based on microsatellites data (Kang *et al.*, 2008). However, previous studies revealed that diploid homosporous fern *Ceratopteris pteridoides* (Hook.) Hieron. (Chen *et al.*, 2010b) and umbrella fern *Sticherus flabellatus* (R. Br.) St. John. (Keiper and McConchie, 2000) predominantly favors inbreeding by AFLP analysis. Reproductive features of rock fern *Asplenium adiantum-nigrum* L. and *Asplenium csikii* Kümmerle & András. are single-spore colonization and subsequent intragametophytic selfing (Ranker *et al.*, 1994; Vogel *et al.*, 1999). Wubs *et al.* (2010) also revealed that homosporous pteridophyte *Asplenium scolopendrium* L. possessed a mixed mating system, with outcrossing when possible and occasional selfing when needed. The t_m - t_s ranged from 0.227 to 0.320 in both populations and at the species level, indicating a low tendency of biparental inbreeding. At the species level, rpm, rps and rt were 0.279, 0.031, and -0.999, respectively. Difference (rps - rpm) of estimate was -0.248. *F* value was -0.133 in species (Table 3). An estimate of the mating system can be obtained from *F*, which measures the deviation of observed genotypic frequencies from those expected at Hardy–Weinberg equilibrium (Wright, 1965). Negative *F* values indicate heterozygote excesses relative to Hardy–Weinberg expectations; a value of 0 signifies Hardy–Weinberg equilibrium (and random mating); and positive values indicate heterozygote deficiencies, probably reflecting high rates of inbreeding (Soltis and Soltis, 1990a). Results of previous studies also indicated that populations of twenty homosporous pteridophytes species with low intragametophytic selfing rates exhibited *F* values near zero (Soltis and Soltis, 1990b). In the study, *F* is negative (-0.133), indicating an excess of heterozygotes and less inbreeding in the populations analyzed. These data indicate that *I. yunguiensis* predominantly favors outcrossing.

Dominant RAPD, ISSR, and AFLP markers have been used to estimate the outcrossing rate in populations of flowering plants (Gaiotto, Bramucci, and Grattapaglia, 1997; Ge and Sun, 1999; Chen *et al.*, 2009; Pometti *et al.*, 2013). In lycophytes, where the mating system is estimated using these dominant markers such as AFLP particularly is limited in comparison to the other species. Mating systems of some fern species such as *Blechnum spicant* and *Dryopteris affinis* ssp. *affinis* had been successfully estimated using AFLP markers (Peredo *et al.*, 2013).

In the present study, employing six primer combinations revealed a large number (160) of variable AFLP loci in *I. yunguiensis*. Although AFLP markers are dominant in most cases, they can detect more variation at the whole genome level than ISSR or RAPD markers, and they may detect variation more efficiently due to large numbers of loci that are readily available for screening (Chen *et al.*, 2009). The dominant behavior of RAPD, ISSR, and AFLP markers provides less information per locus as compared to co-dominant markers (Pometti *et al.*, 2013). This is particularly relevant for applications that require genotype discrimination, as in the case of outcrossing rate estimation (Gaiotto, Bramucci, and Grattapaglia, 1997). Ritland and Jain (1981) demonstrated,

TABLE 4. Genetic diversity of two *Isoëtes yunguiensis* populations studied in China

Population code	Number of polymorphic bands	PPB (%)	Na	Ne	*H*	*I*
PB	114	58.46	1.585 (0.494)	1.332 (0.367)	0.196 (0.198)	0.294 (0.283)
QHH	124	63.59	1.636 (0.482)	1.451 (0.376)	0.258 (0.206)	0.377 (0.295)
Mean	119	61.03	1.611 (0.036)	1.392 (0.084)	0.227 (0.044)	0.336 (0.059)
Species level	160	82.05	1.821 (0.385)	1.491 (0.341)	0.291 (0.172)	0.437 (0.237)

PPB, Percentage of polymorphic bands; Na, Observed number of alleles; Ne, Effective number of alleles; *H*, Nei's gene diversity index; *I*, Shannon's information index; Numbers in parentheses are standard deviations.

through simulation studies, that the limitation associated with dominant loci could be readily overcome by multilocus estimation using a large number of dominant markers with intermediate gene frequencies. The availability of many polymorphic loci provides the opportunity to select the most suitable ones depending on the kind of analysis. Ritland and Jain (1981) described the mixed-mating model, showing that more than five dominant marker loci (with $P = 0.5$) are necessary to reveal low variance in the estimation of outcrossing rate and that dominant loci with allele frequencies close to 0.5 should be preferred to obtain more accurate estimates of mating system parameters. Our study also highlighted that when dominant markers are applied for estimating outcrossing rate, it is imperative to adequately screen primers in order to maximize the probability of amplifying a large number of polymorphic markers in the progeny arrays. The present study indicates that although AFLP markers are dominant and contain lower information content than co-dominant markers such as Allozyme and SSR markers, they are nonetheless suitable for investigating the mating system in *I. yunguiensis*.

The equivalent to crossing in higher plants involves crosses between gametophytes produced by spores from different sporophytes, termed sporophytic outcrossing (Haufler *et al.*, 2016). *Isoëtes yunguiensis* is a heterosporous lycophyte, producing microspores and megaspores, and male and female gametophytes respectively (Shen *et al.*, 2015). Thus, the reproductive nature of *I. yunguiensis* results in either sporophytic selfing (if the sperm and egg come from gametophytes produced by a single sporophyte) or sporophytic outcrossing (if the sperm and egg come from gametophytes produced by different sporophytes) (Haufler *et al.*, 2016).

Survey investigation found that *I. yunguiensis* individuals in the PB and QHH populations mainly grow in the swamp with running water (Table 1). Thus, spore dispersal and swimming sperm of *I. yunguiensis* in flowing water are likely to be frequent. This efficient spore and sperm dispersal might have played an important role in promoting sporophytic outcrossing of *I. yunguiensis*.

Genetic diversity and genetic structure of populations.—160 out of the 195 reproducible bands were polymorphic among 37 individuals in the current study (Table 4). PPB was 82.05% at the species level (Table 4). PPB for a single population ranged from 58.46 % (PB) to 63.59% (QHH), with a mean value of

61.03%. *H* and *I* showed a similar pattern of the genetic diversity between populations (*H* : 0.291, *I* : 0.437, respectively) (Table 4). The results indicate that a relatively high level of genetic diversity was detected within populations and at the species level compared to those obtained from other studies of *Isoëtes* species. Chen *et al.* (2010a) using AFLP data revealed low genetic diversity among populations of *I. hypsophila*, an endangered alpine quillwort in China (PPB: 48.5%, *H* : 0.039, *I* : 0.061, respectively). Kim, Shin, and Choi (2009) using AFLPs also reported low genetic diversity of six endangered *Isoëtes* species from East Asia (Taiwan, Japan, and South Korea) including two diploid species (*I. taiwanensis* [PPB, 33.1%–38.3%] and *I. asiatica* [PPB, 49.0%]), four polyploid species (tetraploid: *I. jejuensis* [PPB, 9.3%–29.3%] and *I. hallasanensis* [PPB, 22.3%]; hexaploid: *I. coreana* [PPB, 1.6%–20.6%] and *I. japonica* [PPB, 5.6%–20.5%]).

Several factors, including the long evolutionary history, historical traits, mutation, genetic drift, mating system, the amount of interpopulational gene flow, the physical features of the habitat itself, as well as its geographic distribution and selection, may determine the population genetic diversity and the genetic structure of populations of ferns (Soltis and Soltis, 1990a). All of these factors are also considered important in seed plants (Loveless and Hamrick, 1984; Soltis and Soltis, 1990a).

Mating system has been reported as an important determinant of genetic variation in plants (Hamrick and Godt, 1996). Outcrossers usually present higher polymorphic loci or number of genotypes per population than inbreeders (Holsinger, 2000). Our results indicated that *I. yunguiensis* predominantly favors outcrossing, resulting in a generally high level of intra- and interpopulation genetic diversity in *I. yunguiensis*.

Apart from the mating system, the level of genetic diversity found in a population is highly dependent on the evolutionary history of the lineage and the populations (Booy *et al.*, 2000). Integrating evidence from fossil records and the current distribution pattern of *Isoëtes* in China, Liu, Gituru, and Wang (2004) proposed that the ancestors of extant *Isoëtes* populations could have reached the Qinghai-Tibet region, indicating that *Isoëtes* occurred on Hengduanshan Mountains in the Holocene (Quaternary) (Bai, Wang, and Liu, 1994). The retreat of the Tethys sea and the uplift of the Qinghai-Tibet Plateau may have contributed to the current distribution pattern of *Isoëtes* in East Asia (Liu, Gituru, and Wang, 2004). *Isoëtes* species in China display a spatial distribution pattern of *I. hypsophila–I. yunguiensis–I. taiwanensis–I. sinensis* from high altitude to low altitude and from west to east (Liu, Gituru, and Wang, 2004). The Yunnan-Guizhou Plateau is located in southwest China, west of the Hengduanshan Mountains. The altitude of the Yunnan- Guizhou Plateau is lower than that of the Qinghai-Tibet Plateau and *I. yunguiensis* is distributed in the Yunnan- Guizhou Plateau in China (Wang *et al.*, 2002). These facts indicate that *I. yunguiens* is a more ancient species (Wang *et al.*, 2002; Chen *et al.*, 2007). Therefore, high genetic diversity in *I. yunguiens* may have resulted from the long-term accumulation of genetic variation through history. Chen *et al.* (2010a) also reported that the strong genetic differentiation

found in *I. hypsophila*, which is an aquatic quillwort endemic to the southeastern Qinghai-Tibet Plateau, may be related to its evolutionary history and breeding system. It is highly likely that these factors maintained a high level of genetic diversity in the present *I. yunguiensis* population.

Inbreeding and genetic drift in small extant populations will inevitably lead to decreasing genetic variability (Ellstrand and Elam, 1993). In the present study, the number of individuals of *I. yunguiensis* at two existing natural populations is no more than 50 (Table 1), indicating the two existing natural populations (PB, QHH) are small populations. Chen *et al.* (2007) reported that recent habitat destruction has greatly reduced the number of individuals in PB population. Although *I. yunguiensis* can resproduce through sporophytic selfing, high levels of intra- and interpopulation genetic diversity in *I. yunguiensis* has been observed, suggesting that recent habitat destruction has not yet resulted in inbreeding or a significant loss of genetic diversity in the *I. yunguiensis* populations (Chen *et al.* 2007). However, given a continuously decreasing population size, genetic diversity is likely to be lost.

Patterns of spore dispersal may also be important in determining genetic variation within populations (Soltis and Soltis, 1990a). Yang *et al.* (2011) reported that the dispersal ability of *Isoëtes* spores or sporophytes along the main water flow direction was stronger than that along the weak flow direction, which suggested water flow had a significant impact on gene flow in *Isoëtes*. Gene flow could potentially prevent the loss of genetic variation within populations and decrease inter-population differentiation (Slatkin, 1987). Because *I. yunguiensis* populations from China grow in swamps and streams, spore dispersal by water flow is probably frequent within and among populations. In addition, although *I. yunguiensis* sperm can swim, they can only survive 11 min (Li *et al.*, 2015), indicating that sperm might move among individuals within populations and be more restricted between populations. Dispersal of spores and moving sperm by water currents may have facilitated extensive gene flow, increasing the rate of outcrossing, and is probably contributing to the high genetic diversity of *I. yunguiensis*.

AMOVA of AFLP genotype data showed that 40.12% of the total genetic variability was attributed to among populations and 59.88% to individual differentiation within populations, indicating highly significant genetic differentiation ($P< 0.001$) between and within populations of *I. yunguiensis* in China (Table 5). Generally, values of F_{st} above 0.25 indicate significant genetic differentiation (Wright, 1978). In this study, the values of G_{st} and F_{st} were 0.223 and 0.401 respectively, also indicating strong genetic differentiation between populations. Moreover, the Nei-Li genetic similarity is 0.75 and UPGMA cluster analysis of the 37 samples from the two groups, the QHH and PB populations (Fig. 2), also suggested significant genetic differentiation between the two extant populations in China.

Based on G_{st} value, Nm between the populations was 1.739. The observed level of gene flow was lower than that in the endangered and endemic aquatic *Isoëtes* in China, such as *I. sinensis* (allozyme analysis: Nm = 4.5062; Chen *et al.*, 2004). The results show that interpopulational gene flow of *I. yunguiensis*

TABLE 5. Analysis of molecular variance (AMOVA) for AFLP data of 37 individuals from two populations of *Isoëtes yunguiensis*

Source of variation	d.f.	SSD	Variance component	Percentage of variation (%)	*P* *
Among populations	1	202.634	10.142	40.12	<0.001
Within population	35	529.906	15.140	59.88	<0.001
Total level	36	732.541	25.282		

d.f., degree of freedom; SSD, sum of squared deviation; *Statistical significance is based on 1000 permutations.

is quite restricted. In plants, gene flow is occasioned by movement of pollen, seeds, spores, and other propagules (Orive and Asmussen, 2000). Dispersal of *Isoëtes* spores is often accomplished via floating leaves (Small and Hickey, 1997). The swimming speed of *I. yunguiensis* sperm is 79μm/s and its life time is only 11 min (Li *et al.*, 2015), indicating long-distance movement of sperm among populations may be restricted. The distance between the two *I. yunguiensis* populations is about 15 km (Fig. 1). Thus, spore dispersal and moving sperm of *I. yunguiensis* is likely to be restricted between populations and might have reduced gene flow. Yang *et al.* (2011) revealed a high level of gene flow via spore dispersal (Nm = 16.66) in neighbor *ex-situ Isoëtes* subpopulations along main water flow and low genetic differentiation among conservation subpopulations ($G_{st} = 0.070$). It is likely that the restricted gene flow between the populations in *I. yunguiensis* may have played an important role in determining the present-day structure of genetic variation in the study populations.

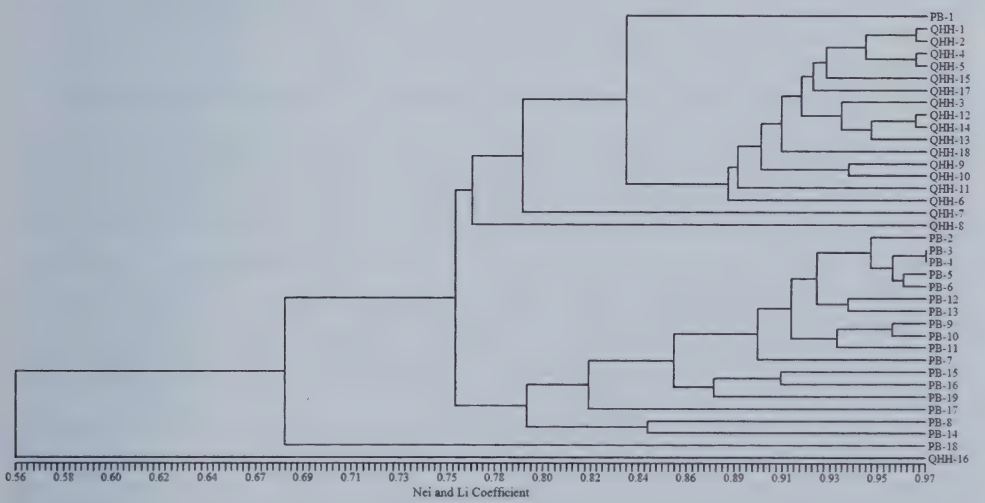

FIG. 2. UPGMA dendrogram of 37 individuals of *Isoëtes yunguiensis* based on AFLP data. The first two or three letters represent the population code, and the number is the individual from each population.

Given that we are considering an endangered species with restricted population sizes at two sites, the sample sizes in the populations varied in the range 18-19 (Table 1), which may have led to bias in statistical analyses.

Implications for conservation.—Both *in situ* and *ex situ* conservation approaches are required for protecting the remaining *I. yunguiensis* populations in China. In recent decades, five *I. yunguiensis* populations (Heilongtan, Songhuaba, Xiaoshao, Shuangshao and Xuandian populations) reported earlier have already been extirpated (Pang *et al.*, 2003) (Fig. 1). The degeneration of primary habitats, deterioration of water quality caused by human activities including farming, grazing, uncontrolled real estate development, and run-off water pollution are identified as the likely key factors responsible for the progressive reduction in the numbers and sizes of *I. yunguiensis* populations (Pang *et al.*, 2003; Liu, Pang, and Wang, 2003; Dong, 2009). The distribution and occurrence of *I. yunguiensis* populations are correlated with water chemistry, pH value, and conductivity as the most important factors (Liu, Pang, and Wang, 2003; Dong, 2009). In addition, Li *et al.* (2015) suggested that an important reason for the endangerment of *I. yunguiensis* in China might be the low production rate of sperm due to water pollution. The change of water environment in habitats has a direct effect on the reproduction of *Isoëtes*. Once the water is polluted seriously, it might interfere with the chemotaxis of sperm and kill them (Li *et al.*, 2015). Potamic and lacustrine water chemical properties are among the principal factors determining the kind, number, and distribution of aquatic plants (Yang and Ye, 2001; Liu, Pang, and Wang, 2003). Therefore, the most appropriate conservation strategy would be to protect more habitats where the species survives. It is worthy of notice that the PB population has been protected by the establishment of nature reserves at their locations. *In situ* conservation was supported by grants from the World Wildlife Fund in 2007. A vital aim of conservation, in addition to habitat preservation, is to maintain a species' existing level of genetic variation in order to maximize its chances for persistence in the face of changing environments (Keiper and McConchie, 2000). Considering the fact that only two extant populations (PB and QHH populations) were found, the high level of genetic diversity within populations and at the species level of *I. yunguiensis* indicate that efforts should be made to conserve every remaining population. However, the two extant populations are both small populations. With the continuing decrease of population size, the genetic diversity will gradually be lost. We suggest that the products from the extant populations should be used for re-establishment of the populations in the same area.

A good knowledge of the mating system of *I. yunguiensis* will provide critical base-line information for developing sustainable management strategies. Considering that outcrossing rates are high, it would be advisable to establish as many *ex situ* conservation sites as possible and transplanting plants between populations to minimize inbreeding and enhance gene flow to preserve the greatest extent of genetic resources within the species.

Conclusion

Overall, our results indicate that *I. yunguiensis* is mostly outcrossing, and has a high level of genetic diversity and highly significant genetic differentiation between and within populations. The combination of high outcrossing rate, accumulation of genetic variation in the evolutionary history of the taxon, evident spore dispersal, inter-individual sperm movement, has probably resulted in the current maintenance of genetic diversity despite small population sizes following habitat destruction in extant *I. yunguiensis* populations. The remaining *I. yunguiensis* populations in China should be a priority for *in situ* conservation.

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Shorter Notes

Ant-Fern Association in *Microgramma megalophylla*.—Association with ants in the genus *Microgramma* C.Presl (Polypodiaceae) is well known in the species previously segregated in *Solanopteris* Copel. [≡*Microgramma* subg. *Solanopteris* (Copel.) Lellinger; Lellinger, American Fern Journal 67:58–60. 1977]. Those epiphytes produce myrmecodomatia, which are hollow lateral rhizomatous sacs inhabited by ants and invaded by the fern roots (Rauh, Akademie der Wissenschaften und der Literatur 5:223–256. 1973; Gómez, Brenesia 4:37–61. 1974). Phylogenetic studies indicate that *Microgramma* species with sac-like myrmecodomatia [*M. bifrons* (Hook.) Lellinger, *M. brunei* (Wercklé ex Christ) Lellinger, *M. fosteri* B.León & H.Beltrán, *M. tuberosa* (Maxon) Lellinger] form a clade (Almeida, Systematic studies in the genus *Microgramma*, PhD. Thesis, 2014). Outside this clade, no other association between ants and *Microgramma* has been reported until now. *Microgramma megalophylla* (Desv.) de la Sota, a species occurring in flooded and non-flooded forested habitats throughout the Amazon lowlands in Bolivia, Brazil, Colombia, Ecuador, Guyana, French Guyana, Peru, and Venezuela, is peculiar in having broad flattened rhizomes up to 45 mm wide that grow attached to tree trunks (Fig. 1A), a character unique in the genus. *Microgramma megalophylla* was found to be associated with ants in plants growing at 4–10 meters above ground in a white-water flooded forest (várzea) at the margins of the Purus River, in Reserva Biológica do Abufari protected area, municipality of Tapauá, Amazonas state (INPA263345, Brazil, *T.E. Almeida 3730*). Seven clumps of plants (growing in different tree hosts) were observed in this population and were all colonized by ants. When detached from the tree, these specimens revealed a hollow cavity formed by the arched rhizome and the roots, the latter forming a network enclosing a tunnel-like chamber against the host tree trunk (Fig. 1B–D). Ants, ant waste, ant carton (cardboard like material built by the ants), and dozens of aggressive ant workers and ant pupae (*Camponotus* sp.) were observed inside this chamber, indicating that *M. megalophylla* housed an ant colony (Fig. 1C–D). Association of *Camponotus* species with ferns have already been documented elsewhere (e.g., Gómez, 1974; Mehltreter, Fern Ecology, p.243. 2010). Further detailed examination of *M. megalophylla* rhizomes in more than 600 herbarium specimens covering all its occurrence range revealed the presence of ant debris and remnant ant tunnels in less than 10% of them. Indications of ant association in, e.g., specimens from Peru (UC1733146, Amazonas, *C. Díaz et al. 7232*; UC1733064, Loreto, *J. Revilla 3346*) and Brazil (UC443238, Amazonas, *Lutzelby 22726*; IAN115067, Amazonas, *E. Oliveira 2271*), confirm that this association is more widespread. The paucity of herbarium specimens with obvious ant associations could indicate that this association is rare but may also be a bias created when the specimen is collected or mounted, when the rhizomes are usually cleaned, and any substrate or debris removed. Association between

FIG. 1. Ant-fern association in *Microgramma megalophylla*. **A.** Plant habit, showing wide flattened rhizomes. **B.** Lower surface of rhizome showing a net of roots, associated with ant nest carton. **C.** Detail of the lower surface of rhizome, showing ant workers and pupae. **D.** Cross section of the rhizome showing ants and pupae occupying the tunnel formed by the rhizome, the network of roots, and the ant carton. (scale bars = 1 cm)

ants and epiphytes is common in the upper canopy of flooded forests (Majer and Delabie, Insectes Sociaux 41:343–359. 1994). Moreover, the association observed in *M. megalophylla* is similar to that found in *Microgramma* sect. *Solanopteris*, differing in the absence of the sac-like myrmecodomatia. In fact, in *M. brunei*, even when myrmecodomatia were available most *Azteca* ants still colonized the rhizome and roots with the myrmecodomatia being used by the queen as brooding chambers (Gómez, 1974). Huxley (Biological Reviews 55:321–340. 1980) mentioned an irregular association of ants with *Polypodium schomburgkianum* Kunze (≡*Microgramma megalophylla*), based on a report by Spruce (Notes of a Botanist on the Amazon and Andes, pp.384–412. 1908), but the material cited by Spruce refers to *M. bifrons*. The interactions between ants and epiphytic ant-plants are generally considered to arise for multiple reasons for both partners: ants receive shelter and/or food from the plant and the plants are defended and/or absorb nutrients from organic debris left by ants. Ant waste as a source of nutrient is likely to be an important gain, since epiphytes are expected to have less access to nutrients (Huxley, 1980). The first aspect –

plant protection – has been reported for *M. brunei* (Gómez, 1974), whereas evidence for plant nutrient acquisition in ant-plant interactions is known in ferns only for *Lecanopteris* (Gay, Biological Journal of the Linnean Society 50:221–233. 1993), and *Antrophyum* (Watkins *et al.*, New Phytologist 180:5–8. 2008) with most epiphytic ant ferns remaining untested. In the plants reported here, the trichome-covered roots of *M. megalophylla* grew into the ant chamber amongst the ant-carton and waste (Fig. 1B–D) rather than spread over the tree trunk, supporting a view the association is related to plant nutrition. The roots in *M. megalophylla* potentially play a similar role to the roots growing inside the myrmecodomatia in subg. *Solanopteris*, where a high level of nitrogen and phosphoric compounds in the debris contents left by the ants was reported (Gómez, 1974). Another ant-fern, *Lecanopteris mirabilis* (C.Chr.) Ching, also has flattened rhizomes (Gay, Botanical Journal of the Linnean Society 106:199–208. 1991) like *M. megalophylla*, but considerably wider. Other members of the genus *Lecanopteris* have more complex myrmecodomatia including tunnels and chambers within the hollow rhizomes (Gay, Botanical Journal of the Linnean Society 113:135–160. 1991). Modification of flattened rhizomes into ant chambers may have fostered stronger mutualistic associations in *Microgramma* and *Lecanopteris* and may explain the unusual autapomorphy of wide flattened rhizomes in *M. megalophylla*. Supporting this hypothesis is the placement of *L. mirabilis* as sister to other *Lecanopteris* species with more derived rhizome structures (Haufler *et al.*, Systematic Botany 28:217–227. 2003). However, *M. megalophylla* is not closely related to *Microgramma* subg. *Solanopteris* (Almeida, 2014) and it is potentially a separate origin of ant association. Further investigations are needed to clarify whether the interaction between ants and *M. megalophylla* is opportunistic or mutualistic, and if the plants are actively acquiring nutrients harvested from the landscape by the ants. Limited nutrient availability could be a driver in mutualistic adaptations in ferns (Gay, 1993), and associations like the one described here might help us understand the physiological and environmental pressures that led to the evolution of such intricate mutualistic associations as the ones presented by *Microgramma* subg. *Solanopteris*.—Thaís Elias Almeida, Universidade Federal do Oeste do Pará, Herbário HSTM, Avenida Marechal Rondon, s.n., Santarém, Pará, Brazil. CEP: 68040-070. E-mail: blotiella@gmail.com.

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COVER CAPTION: *Microgramma megalophyla.* Top: fertile leaf (left), leaf apex (right). Bottom: rhizomes. Photo credits: Leandro L. Giacomin (top, right); Thaís Elias Almeida (all others).