ORIGINAL ARTICLE

Reproductive isolation between diploid and tetraploid cytotypes of *Libidibia ferrea* (= *Caesalpinia ferrea*) (Leguminosae): ecological and taxonomic implications

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Abstract Polyploidy is the most common chromosomal mechanism involved in the evolution of plants. However, the emergence of polyploid individuals does not guarantee the establishment of a new lineage, and the relationship between polyploidy and reproductive biology is therefore relevant. *Libidibia ferrea* is a legume tree that has diploid and tetraploid populations. In this work we analyse the reproductive biology of the species to verify the degree of reproductive isolation between the two cytotypes. Observations on phenology, floral morphology, biology, and visitors, breeding system, reproductive success and reproductive isolation were made for both cytotypes in two municipalities of northeastern Brazil. Cytotypes differed for all morphometric parameters analysed, with tetraploids exhibiting higher mean values than diploids. Both cytotypes had the same effective pollinators (native bees from the genera *Centris* and *Xylocopa*, and the introduced *Apis mellifera*). However, since stamens of diploids and tetraploids were of different size, it is possible that spatial separation occurs when pollen of each cytotype is deposited on a bee's body. Diploids were self-incompatible (ISI = 0) and exhibited high fruit-set after intraploidy crossing (20 %), whereas tetraploids were self-compatible (ISI = 0.47) and set only 3.9 % of fruits after intraploidy crossing. Both cytotypes showed low fruit- and seed-set under natural conditions, and there was no fruit-set after crosses between them. Data are discussed in relation to the establishment of polyploids, to the pattern of distribution of the species and to the taxonomic implications. The cytotypes of *L. ferrea* should be considered as distinct species, since they are reproductively isolated from each other.

Keywords Autopolyploidy · Biosystematics · Caatinga · Fabaceae · Polyploidy · Reproductive biology

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Introduction

Polyploidy, the multiplication of complete sets of chromosomes, is an important phenomenon in the evolution of many lineages of eukaryotes (Ramsey and Schemske 1998). It is the most frequent cytogenetic change and the most common chromosomal mechanism involved in the evolution of plants (Guerra 2008); it has previously been estimated to have occurred in up to 70 % of all angiosperms (Masterson 1994), but recent phylogenomic analyses indicate that probably all angiosperms have at least one round of ancient polyploidy in their ancestry (Jiao et al. 2011).

By affecting the entire genome, several phenotypic traits can be simultaneously influenced by polyploidy (Husband et al. 2008). Because of its most immediate effect, i.e., an increase in cell size, multiplication of the genome can result in morphological, physiological and ecological alterations (Stebbins 1940; Levin 2002). Some of these

changes may be adaptive and allow polyploids to occupy new ecological niches (Ramsey and Schemske 1998; Levin 2002).

However, the emergence of polyploid individuals does not guarantee that a new lineage will become established (Fawcett and Van de Peer 2010), and the relationship between polyploidy and reproductive biology is therefore relevant. Although there are examples of different cytotypes of the same species occupying the same area, diploid and polyploid cytotypes usually have distinct geographical distributions (Thompson and Lumaret 1992). Levin (1975) has argued that, in addition to the ecological differences that may at first go unnoticed by an observer, this pattern of distribution can be explained by the relationship between reproductive success and the frequency of a cytotype. Levin's "Minority Cytotype Exclusion" model states that because crosses between diploid and tetraploid zygotes generate triploids that are almost completely unviable or sterile, the lower the frequency of a cytotype is, the lower the proportion of compatible crosses and, thus, the lower its reproductive success. This disadvantage is amplified in subsequent generations, which become smaller each time, and ultimately the minority cytotype is eliminated from the population.

Several factors can counteract or slow the decrease in frequency of a minority cytotype (Husband 2000; Fowler and Levin 1984; Felber 1991; Rodríguez 1996). These include a high production rate of unreduced gametes (which will increase the formation of new polyploid individuals); niche separation; the tolerance and frequency of self-pollination; an increased dispersal of pollen and seeds (which could increase the reproductive success of the minority cytotype); and stochastic effects. Some of these factors are independent of polyploidy, but two could be greatly affected by the increase in ploidy level and may be subject to natural selection. These are niche separation and the tolerance and frequency of self-pollination, which are both related to the reproductive biology of a species.

Polyploidy may have several effects on plant reproduction (see Levin 2002 for a review). The most common alterations include the breakdown of self-incompatibility, and the change from sexual to asexual reproduction via apomixis (Levin 1983; Barringer 2007); changes in a species' sexual system, through the collapse of self-incompatibility followed by increased inbreeding depression and possibly the subsequent invasion of unisexual mutant plants that are incapable of autogamy (see Miller and Venable 2000); phenological differences due to delayed or advanced flowering periods (e.g. Husband and Schemske 2000); and changes in floral attraction due the increased size and quantity of resources, which have important consequences for plant-pollinator interactions (e.g. Segraves and Thompson 1999). All of these changes

can affect both niche separation and the tolerance and frequency of self-pollination. Therefore, alterations to a plant's reproductive biology are likely to be highly significant in the establishment of a polyploid lineage.

Beltrão and Guerra (1990) have reported the occurrence of both diploid ($2n = 24$) and tetraploid ($2n = 48$) individuals in *Libidibia ferrea* (Leguminosae–Caesalpinioideae), and based on the karyotype similarities between the cytotypes, the authors suggested that the tetraploid form originated through autopolyploidy. *Libidibia ferrea*, a tree native to eastern and northeastern Brazil, has a wide geographical distribution and great morphological variability, an attribute that has led to the creation of several infraspecific classifications (Lewis 2005; Queiroz 2009). It is possible that this morphological variation is partly caused by ploidy variation in the species. The objective of this study was to analyse various aspects of the reproductive phenology, floral morphology and reproductive biology of *L. ferrea*, with comparisons made between diploid and tetraploid individuals, and to verify the degree of reproductive isolation between the two cytotypes.

Materials and methods

Species studied

Libidibia (DC.) Schltdl. is a neotropical genus of six to eight species of trees or shrubs that occur mostly in seasonally dry forests and that were previously treated under *Caesalpinia* (Lewis 2005). Recently, Queiroz (2009) proposed the new combination for three varieties of the only species native to Brazil, *Libidibia ferrea* (Mart. ex Tul.) L.P. Queiroz, which is popularly known as jucá or pau-ferro. The species occurs in the east and northeast in seasonally dry forests and in Caatinga vegetation, a specific type of seasonally dry forest that displays the short tree stature and xeromorphic features commonly found in the semiarid regions of Brazil (Lewis 2005; Queiroz 2009). Four morphological varieties of the species are currently recognised: *Libidibia ferrea* var. *ferrea* (Benth.) L.P. Queiroz, *L. ferrea* var. *glabrescens* (Benth.) L.P. Queiroz, *L. ferrea* var. *parvifolia* (Benth.) L.P. Queiroz (Queiroz 2009) and *Caesalpinia ferrea* var. *leiostachya*. *Caesalpinia ferrea* var. *leiostachya* is restricted to the Atlantic Forest of Rio de Janeiro (Rizzini and Mattos-Filho 1968), whereas *L. ferrea* var. *parvifolia* occurs both in the Atlantic forest and the Caatinga; *L. ferrea* var. *ferrea* and *L. ferrea* var. *glabrescens* are restricted to the Caatinga (Queiroz 2009). Therefore, the Caatinga is the only region where three of the four varieties can be found.

Given that the dimensions of tetraploid organisms are generally larger than those of diploids (Stebbins 1940) and

that one of the traits distinguishing the varieties of *L. ferrea* is the size of the leaflets (Queiroz 2009), it is possible that *L. ferrea* var. *parvifolia*, which has leaflets smaller than 0.9 cm length (Queiroz 2009), consists of diploids, whereas *L. ferrea* var. *ferrea* and *L. ferrea* var. *glabrescens*, which have leaflets greater than 1.5 cm length (Queiroz 2009), consists of tetraploids. The basic difference between the latter varieties lies in the density of the trichomes that cover the leaflets, which are pubescent in *L. ferrea* var. *ferrea* and glabrous in *L. ferrea* var. *glabrescens*. This work included the varieties *L. ferrea* var. *ferrea*, *L. ferrea* var. *parvifolia* and *L. ferrea* var. *glabrescens*, as described below (see "Study area").

Study area

The information presented in Beltrão and Guerra (1990) and an initial analysis of the *L. ferrea* specimens in the Herbarium of the Federal University of Pernambuco (UFP) indicated that the two cytotypes and all three morphological varieties of the species that occur in the Caatinga can be found in Pernambuco State, Brazil. Thus, the voucher specimens of *L. ferrea* collected in this State and available at the UFP Herbarium were analysed to provide a preliminary idea about distributions. Based on the relationship between polyploidy and increased dimensions of organisms outlined above, the samples were initially classified as follows: (1) potentially diploid (with leaflets up to 0.9 cm long) or (2) potentially tetraploid (leaflets longer than 1.5 cm). According to this preliminary survey, most regions within Pernambuco State showed only one cytotype and the following trend was observed: potentially diploid individuals occurred in areas of dry forests located near the coast (e.g., the Pedra da Mata Forest in Nazaré da Mata) or in areas with higher elevations within the state (e.g., the Catimbau National Park in Buíque), whereas potentially tetraploid individuals were located throughout the interior of the state in areas of Caatinga with elevations below 800 m. Considering the morphological varieties, the distribution of *L. ferrea* var. *parvifolia* coincided with that of the assumed diploid individuals, while *L. ferrea* var. *ferrea* and *L. ferrea* var. *glabrescens* were found within the range of the assumed tetraploid individuals. However, although *L. ferrea* var. *ferrea* and *L. ferrea* var. *glabrescens* were occasionally found in simpatry, the *L. ferrea* var. *ferrea* presented a wider range than *L. ferrea* var. *glabrescens*, which was restricted to a few localities within the State (e.g. Alagoinha and Venturosa municipalities).

Following this initial step, field observations and seed collections were conducted between 2007 and 2008. This material was used to analyse the ploidy level of the individuals sampled (the method employed is described below) and to determine the cytotypes that occur at each location.

Voucher specimens of diploid and tetraploid individuals were deposited in the UFP Herbarium.

After determining the ploidy level of individuals from various sampling sites, a study area for detailed investigation of the reproductive biology of both cytotypes was selected between the municipalities of Alagoinha (08°29′S, 36°49′W), which had tetraploid individuals, and Poção (08°11′S, 36°42′W), where most individuals are diploid, but some tetraploid individuals also occur. The diploids that were found were consistent with the description of *L. ferrea* var. *parvifolia*, while some tetraploids fit the description of *L. ferrea* var. *ferrea* and others fit that of *L. ferrea* var. *glabrescens*.

The municipalities selected are situated about 200 km from the coast, in the semiarid region of Pernambuco State, with 30 km between each location. Both areas are located in the Maciço da Borborema, but at different altitudes and in varying ecological conditions: Alagoinha is approximately at 700 m asl, whilst Poção is at a higher elevation (approximately 1,030 m asl) with higher humidity and lower temperatures, which influence the vegetation (Araújo-Filho et al. 2000). Alagoinha has a higher average annual temperature (21.6 °C) and lower annual rainfall (597 mm) than Poção (19.5 °C and 753 mm,) (LAMEPE (Laboratório de Meteorologia de Pernambuco) 2010). There are two well-defined seasons, a wet and a dry one. In Alagoinha, the rainy season occurs for 6 months, from February to July, while the rainy season in Poção lasts 7 months, from January to July (LAMEPE 2010). The typical vegetation in Alagoinha consists of hypoxerophytic Caatinga, and that of Poção includes both hypoxerophytic Caatinga areas and 'Brejos de Altitude' (semideciduous upland forests), depending on which side of the mountain the area is located (Araújo-Filho et al. 2000). Although the local vegetation has been extensively cut for fuel-wood and livestock farming (Araújo-Filho et al. 2000), individuals of *L. ferrea* are found throughout the pastures because this species has been retained to provide shade and forage for livestock.

Cytogenetic analyses

The ploidy level of all individuals used in this study was determined by analysing the chromosome numbers of seedlings derived from seeds collected from each individual, using one to three seedlings per tree sampled. The seeds were scarified with sulphuric acid and germinated in petri dishes with filter paper soaked in distilled water. After germination, the root tips were pre-treated with 8-hydroxyquinoline (0.002 M) at 10 °C for 24 h, fixed in ethanol:acetic acid (3:1, v/v) at room temperature for 2–24 h and stored at −20 °C. None of the seeds that germinated were polyembryonic.

The chromosome numbers and morphologies of both cytotypes of *L. ferrea* were analysed. To prepare the slides, root tips were washed in distilled water and digested in a solution of 2 % cellulase (Onozuka)-20 % pectinase (Sigma) at 37 °C for 90 min. The meristem was macerated in a drop of 45 % acetic acid, and the cover slip was removed with liquid nitrogen. The material was stained with DAPI (2 μg/ml) for 60 min (Barros e Silva and Guerra 2010). The slides were mounted in glycerol-McIlvaine buffer at pH 7.0 (1:1) and aged for 3 days prior to analysis under a Leica DMLB fluorescence microscope. Cell images were acquired using a Cohu CCD video camera and the Leica QFISH software and subsequently edited in Adobe Photoshop CS3 version 10.0.

Phenology, morphology and floral biology

Bi-weekly visits were made to the study area between December 2007 and March 2008 and between December 2008 and March 2009 to define the period and duration of the flowering episodes of individuals and populations of *L. ferrea*. During the fruit-collecting trips, conducted over several months in 2007 and 2008, we were able to determine the period of fruit dispersal of the species. Observations of both diploid and tetraploid individuals were made on morphology, flower type, the sexual system and flower-opening time. The following parameters were evaluated for five individuals of each cytotype: (1) horizontal and vertical length of the corolla; (2) total length of the stamens, ovary and style; (3) partial length of the stamens and pistil combined, measured from the apex of the anthers and stigma to the curved portion of the standard petal (flag or vexillum), which corresponds to the landing platform for visiting bees; (4) the number of ovules per flower; (5) pollen viability; and (6) the equatorial diameter of the pollen grains. For the first three parameters, we used the average of three flowers per individual, and for the last three, one flower per individual was used. Pollen viability was evaluated by cytoplasmic staining, following the method proposed by Alexander (1980), of 200 grains per slide/anther and four slides per individual. The equatorial diameter of the grains was measured with an ocular micrometre, and 20 pollen grains per slide/anther were evaluated. As the only difference between the flowers of tetraploids of the *glabrescens* and *ferrea* varieties is the pilosity of the ovary (Queiroz 2009), their measurements were grouped in the tetraploid category.

Floral visitors

The floral visitors were observed in six tetraploid individuals during the 2008 flowering period, and the visitors to five diploid and five tetraploid individuals were observed during the 2009 flowering period, resulting in about 160 observation hours between January and February of each year. According to their behaviour at the flowers, the visitors were classified as follows: (1) effective pollinator—when a visitor collected nectar and touched the flower's reproductive parts with the ventral part of its body; (2) occasional pollinator—when a visitor collected pollen, and because of the proximity between the anthers and stigma, pollination could occur, or when the visitor collected nectar and occasionally touched the reproductive parts of the flower; and (3) robber—when a visitor collected nectar but never touched the stigma or anthers. Specimens were collected for later identification and deposited as vouchers in the entomological collection of the Laboratory of Floral and Reproductive Biology at the Federal University of Pernambuco, Recife, Brazil. When possible, photographs were taken to support the description of the observed behaviour.

Breeding system and reproductive isolation between cytotypes

The breeding system of individuals of the two cytotypes, and whether any reproductive isolation occurs between the diploid and tetraploid plants were analysed through controlled pollination treatments conducted during reproductive episodes between January and February of 2008 and 2009. Six tetraploid individuals from Alagoinha were used in 2008, and five diploid individuals from Poção and five tetraploid individuals from Alagoinha (including two used in the previous year) were used in 2009. Tetraploid individuals belonged to both *glabrescens* and *ferrea* varieties and were used as both pollen donor and receptor. All individuals received hand self-pollination and cross-pollination treatments, which were performed with newly opened flowers that had been previously enclosed in semi-permeable bags during the pre-anthesis stage. After pollination, the flowers were re-bagged for 2 days and then uncovered and monitored for fruit-set.

The following treatments were used: (1) hand self-pollination ($N = 160$ flowers, with 2–19 flowers per plant in 2008 and 10 flowers per plant in 2009); (2) cross-pollination between individuals of the same cytotype (intraploidy) ($N = 201$ flowers, with 5–21 flowers per plant in 2008 and 12 flowers per plant in 2009); and (3) cross-pollination between diploid and tetraploid individuals (interploidy) ($N = 120$ flowers, 12 flowers per plant in 2009). For cross-pollinations, flowers were emasculated and each ovulate parent received pollen from six donors in 2008 (one to five flowers of each ovulate parent per pollen donor). In 2009 each ovulate parent received pollen from four diploid and four tetraploid donors (three flowers of each ovulate parent per pollen donor). From the results, we calculated the index of self-incompatibility (ISI), i.e. the ratio between the

percentage of fruit-set per self-pollination and the percentage of fruit-set per cross-pollination (modified from Zapata and Arroyo 1978), where values of up to 0.2 indicate a self-incompatible species, and values above 0.2 indicate a self-compatible species.

Reproductive success

The natural fruit set on the individuals used in the controlled pollination treatments was evaluated in 2009 by marking and monitoring 30 individual flowers (10 flowers per day for 3 separate days), under natural conditions. With the values obtained, we calculated the Reproductive Efficacy, which is the ratio of the percentage of fruits formed under natural conditions to that of fruit formed by hand cross-pollinations (sensu Zapata and Arroyo 1978).

We also compared the average number of seeds per fruit in the following categories: (1) intact seeds, (2) predated seeds, (3) aborted seeds and (4) total seeds. For this evaluation, we used fruits collected from different locations in Pernambuco between 2008 and 2009. For diploids, we used the fruits of individuals in the municipalities of Buíque, Caruaru, Cimbres, Itamaracá, Jaboatão dos Guararapes, Nazaré da Mata, Poção, São Lourenço da Mata and Vicência ($N = 15$ individuals, 6–10 fruits per individual, for a total of 135 fruits). The fruits of tetraploids were from individuals in the municipalities of Alagoinha, Buíque, Caruaru, Poção, Serra Talhada and Venturosa ($N = 20$ individuals, 4–10 fruits per individual, for a total of 182 fruits). Data from natural fruit- and seed-sets were used to calculate the Pre-Emergent Reproductive Success of the two cytotypes, which is the product of the fruit/flower and seed/ovule ratios (sensu Wiens et al. 1987).

Statistical analyses

To evaluate the differences among the floral morphology, size and pollen viability parameters and the number of seeds of diploids and tetraploids, the data were compared using the *t*-test, with $\alpha = 0.05$. The mean numbers of seeds for both cytotypes in the "intact", "predated" and "aborted" categories were compared with ANOVA, with $\alpha = 0.05$, by an a posteriori Tukey test. The data normality and homogeneity of variances were checked using the Shapiro-Wilk and Levene tests, respectively (Sokal and Rohlf 1995). All tests were performed using the Statistica 7.0 software.

Results

Cytogenetic analyses

Four out of the six populations of *L. ferrea* analysed had only one cytotype. One population was diploid (Nazaré da Mata, $N = 2$ individuals), three proved to be tetraploid (Alagoinha, Pesqueira and Serra Talhada, $N = 12$ individuals) and two were mixed (Buíque and Poção, $N = 10$ individuals).

Both cytotypes of *L. ferrea* had small chromosomes (>3.18 μm), including metacentric (M) and acrocentric (A) chromosomes, with a basic karyotype formula of $x = 8$ M $+ 4$A. We observed diploid individuals with $2n = 2x = 24$ (16 M $+ 8$A) (Fig. 1a) and tetraploid individuals with $2n = 4x = 48$ (32 M $+ 16$A) (Fig. 1b). A small number of endopolyploid cells were found in most root tips analysed.
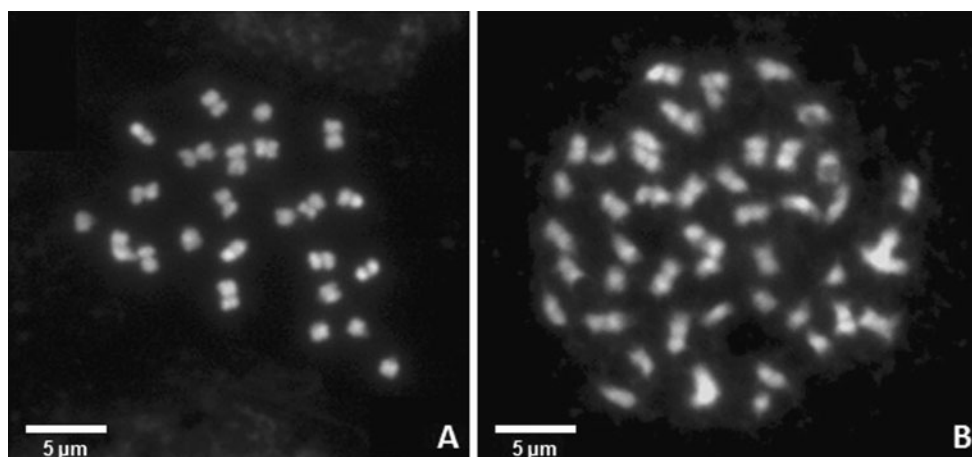

Fig. 1 Mitotic metaphases of diploid (**a**) and tetraploid (**b**) individuals of *Libidibia ferrea* (Leguminosae–Caesalpinioideae) occurring in Pernambuco State, northeastern Brazil

Phenology, morphology and floral biology

At the study sites, *Libidibia ferrea* flowered from December to February, which corresponds to the transition between the dry and wet seasons. The species flowered massively, and although the populations flowered for about 3 months, the individual reproductive period lasted just over a month for most trees. Thus, both diploids and tetraploids were asynchronous, within and between cytotypes. In Poção, where the two cytotypes were found together, the flowering of the few tetraploid individuals occurred during the period in which the diploids were in bloom. The fruiting period of the species was long, nearly 9 months, and the indehiscent fruits were released between September and November, at the peak of the dry season.

The inflorescences are panicles and the flowers, which have a slightly sweet odour, are hermaphroditic, pentamerous and zygomorphic, a standard flower type sensu Faegri and Pijl (1979). The calyx is light green, and the corolla is yellow with small red blotches on the lower half of the standard petal forming a nectar guide (Fig. 2a, b). The ten stamens have longitudinally dehiscent anthers and encircle the style (Fig. 2a, b) and are arranged in two whorls, one inner and one outer. The stamens, which are tightly arranged with densely pilose filaments, form a protective barrier to the nectar, which is produced in the hypanthium. Nevertheless, the base of the central adaxial stamen is separated from the two lateral stamens to form two small windows or slits, through which the pollinators are able to obtain nectar; these openings are themselves protected by the claw of the standard petal. The superior ovary has a short gynophore and at the tip of the relatively long style is a chambered stigma, the opening of which is surrounded by a ring of hairs or papillae that function as a pollen comb. The stigma is of the wet papillate type (sensu Owens 1989). In some flowers, especially of the diploids, the pistil was reduced or stunted, and although we did not quantify the frequency and position of this kind of flower, it seemed to be a non-prevalent and a random phenomenon, with no clearly observed pattern.

Despite the general similarities between the morphology of the flowers of diploids and tetraploids, some significant differences were observed, including a more intense colour and a greater opening of the petals of the tetraploids, as well as a larger and more conspicuous standard petal (Fig. 2a, b). In addition, all morphometric parameters that
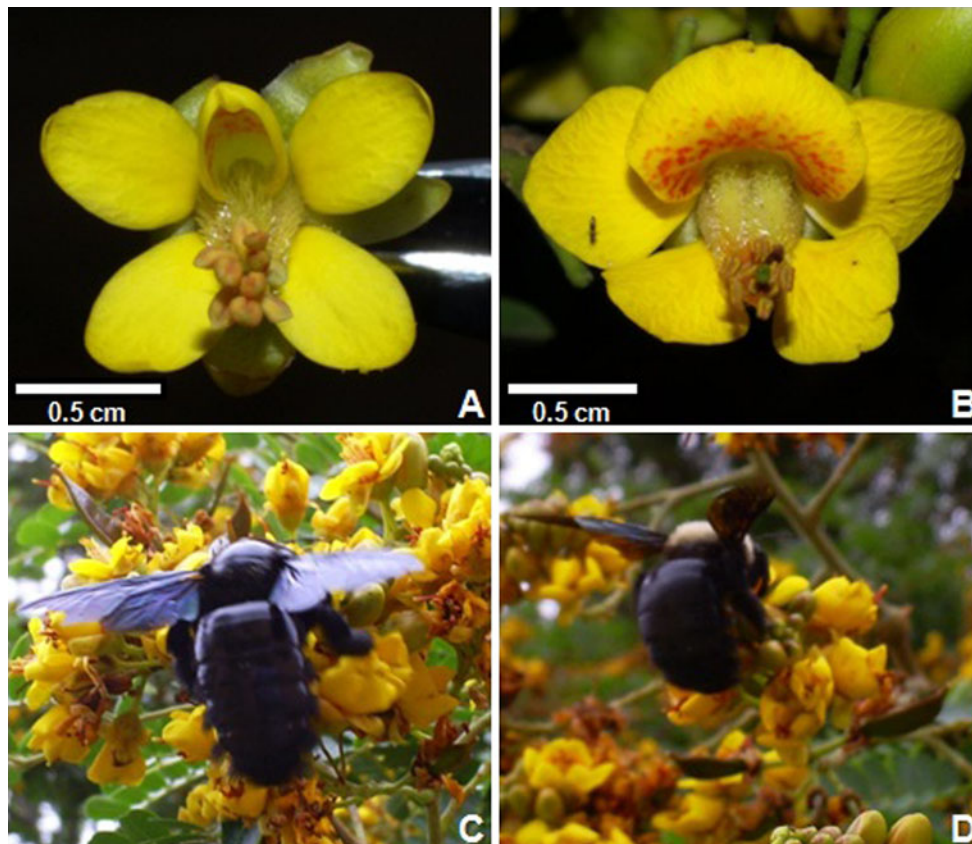

Fig. 2 Flowers of diploid (**a**) and tetraploid (**b**) individuals of *Libidibia ferrea* (Leguminosae-Caesalpinioideae) occurring in Pernambuco State, northeastern Brazil; *Xylocopa frontalis* (**c**) and *Xylocopa grisescens* (**d**) pollinating flowers of a tetraploid individual

were analysed, such as the corolla and ovary length and the number of ovules, differed significantly in mean between the two cytotypes, with the tetraploids having larger values (Table 1). There was also a significant difference between pollen grain size, with diameters of 6.90 ± 0.16 μm in diploids and 8.68 ± 0.35 μm in tetraploids ($t = -9.2941$, $p < 0.0001$), but not between pollen viability, which was 97.16 ± 2.31 % for diploids and 98.4 ± 1.49 % for tetraploids ($t = -1.4024$, $p = 0.1778$).

Both cytotypes exhibited diurnal anthesis that lasted approximately 12 h. The flowers begin to open early in the morning and were completely opened by about 0600 h. The anther dehiscence began at 0700 h, and they were all open nearly 2 h later, when the pollen, which was slightly sticky, was fully available to visitors. In the late afternoon (ca. 1800 h), the standard petal began to curve toward the centre of the flower and by the next day, all of the petals were closed, surrounding the androecium, while growth of the style, which had extended in length from the previous day, resulted in the stigma remaining exposed.

Floral visitors

The effective pollinators of both diploids and tetraploids were medium- to large-sized bees of the genera *Centris* and *Xylocopa* (Fig. 2c, d) and the introduced honey bee, *Apis mellifera* (Table 2). When landing on the flower to seek nectar, the bees touched the anthers and stigma with the ventral part of their bodies, thus effecting pollination. Visits were recorded during the entire period of floral anthesis. The bee *Trigona spinipes* had two types of behaviour: it acted as a nectar robber by perforating the calyx without contacting the reproductive parts of the flower and it could also act as an occasional pollinator by contacting the stigma during pollen collection. The bees

Frieseomelitta doederleini and *Plebeia* sp. were observed to collect pollen, occasionally contacting the stigma, and were considered occasional pollinators. Some hummingbirds (Table 2) visited the flowers of *L. ferrea*, particularly the larger flowered tetraploids. They were considered occasional pollinators because their visits to the flowers were less frequent than those of bees, they were not observed at all individuals, they visited fewer flowers per tree because of the agonistic behaviour amongst them, and they had only occasional contact with the reproductive parts of the flower.

A large number of flowers in both diploids and tetraploids showed signs of damage or the presence of florivores that could damage the reproductive structures. In most of these flowers, the florivores were larvae of Lepidoptera, and their food often consisted of tissue of the ovary and ovules, which were partially or entirely destroyed. As a result of this interaction, many wasps were observed inspecting the flowers inside and out, especially flowers post-anthesis, in search of larvae to eat.

Breeding system and reproductive isolation between cytotypes

The diploid individuals of *L. ferrea* did not develop fruit after self-pollination but had a relatively high percentage of fruit-set after intraploidy cross-pollination (Table 3). The tetraploid individuals of both var. *ferrea* and var. *glabrescens* developed a few fruits (around 2 %) after self-pollination (Table 3), and a similar value was found for the fruit-set after intraploid cross-pollinations, i.e. <4 % (Table 3). It is important to point out that there was fruit-set after crosses between the two tetraploid varieties examined. Thus, with respect to the breeding system, diploids were self-incompatible, and tetraploids were

Table 1 Floral morphometry (mean ± SD) of diploid and tetraploid cytotypes of *Libidibia ferrea* (Leguminosae–Caesalpinioideae) occurring in Pernambuco State, northeastern Brazil

Different letters on the same line mean significant difference

[a] Partial length of stamens and pistils combined, measured from the apex of the anthers and stigma to the curved portion of the standard petal (flag or vexillum), which corresponds to the landing platform for visiting bees during the visit

Parameter (mm)	Diploids (mean ± SD)	Tetraploids (mean ± SD)	t and p
Corolla			
Horizontal length	12.76 ± 1.03 a	16.91 ± 0.62 b	$t = -7.6905$; $p = 0.0001$
Vertical length	10.65 ± 1.33 a	13.95 ± 0.94 b	$t = -4.5056$; $p = 0.0019$
Ovary			
Length	3.04 ± 0.29 a	4.60 ± 0.65 b	$t = -4.8941$; $p = 0.0012$
Diameter	1.06 ± 0.09 a	1.44 ± 0.23 b	$t = -3.4403$; $p = 0.0088$
Ovule number	7.40 ± 0.55 a	10.00 ± 0.71 b	$t = -6.5$; $p = 0.0002$
Style			
Length	6.40 ± 0.55 a	8.42 ± 1.18 b	$t = -3.4775$; $p = 0.0083$
Diameter	0.44 ± 0.05 a	0.52 ± 0.04 b	$t = -2.5298$; $p = 0.0353$
Stamens			
Length	8.13 ± 0.47 a	10.3 ± 0.82 b	$t = -7.3564$; $p < 0.0001$
Landing platform[a]			
Length	7.60 ± 0.62 a	8.97 ± 0.30 b	$t = -4.4004$; $p = 0.0023$

Table 2 Floral visitors of diploid and tetraploid individuals of *Libidibia ferrea* (Leguminosae–Caesalpinioideae) occurring in Pernambuco State, northeastern Brazil

Species	Collected resource	Visitor activity
Bees		
Hymenoptera-Apidae		
Apis mellifera Linnaeus, 1758	Nectar	Effective pollinator
Centris aenea Lepeletier, 1841	Nectar	Effective pollinator
C. fuscata Lepeletier, 1841	Nectar	Effective pollinator
Frieseomelitta doederleini (Friese, 1900)	Pollen	Occasional pollinator
Plebeia sp.	Pollen	Occasional pollinator
Trigona spinipes (Fabricius, 1793)	Nectar/pollen	Robber/occasional pollinator
Xylocopa frontalis (Olivier, 1789)	Nectar	Effective pollinator
Xylocopa grisescens Lepeletier, 1841	Nectar	Effective pollinator
Hummingbirds		
Apodiformes-Trochilidae		
Chlorostilbon lucidus (Shaw, 1812)	Nectar	Occasional pollinator
Eupetomena macroura (Gmelin, 1788)	Nectar	Occasional pollinator

Table 3 Results of controlled and natural pollination treatments, Index of Self-Incompatibility (modified from Zapata and Arroyo 1978), Reproductive Efficacy (sensu Zapata and Arroyo 1978) and Pre-emergent Reproductive Success (sensu Wiens et al. 1987) in diploid and tetraploid individuals of *Libidibia ferrea* (Leguminosae-Caesalpinioideae) occurring in Pernambuco State, northeastern Brazil

Treatments and related indexes	Diploids		Tetraploids	
	Flowers (N)	Fruits (N)/success	Flowers (N)	Fruits (N)/success
Hand self-pollination	50	0	110	2/1.81 %
Intraploidy cross-pollination	60	12/20 %	141	7/3.86 %
Interploidy cross-pollination	60	0	60	0
Natural pollination	150	5/3.3 %	150	4/2.7 %
Index of Self-Incompatibility	0		0.47	
Reproductive Efficacy	0.16		0.69	
Pre-Emergent Reproductive Success	0.015		0.006	

considered self-compatible (see Indexes of Self-Incompatibility in Table 3). None of the crosses between plants of different ploidy levels set any seed (Table 3).

Reproductive success

Both diploids and tetraploids had low natural fruit-set, <4 % (Table 3). However, because diploids produced more fruits than tetraploids in the intraploidy cross-pollination treatment (Table 3), the Reproductive Efficacy of the two cytotypes varied greatly, with 0.16 in diploids and 0.69 in tetraploids (Table 3).

The average number of seeds per fruit was significantly different between the cytotypes: diploids had higher numbers of seeds than tetraploids (4.41 ± 1.37 vs. 3.19 ± 1.23 seeds per fruit, respectively, $t = 2.7059$, $p = 0.0108$). When seeds were analysed by category (intact, predated and aborted), there was a difference between the cytotypes ($F = 5.6296$, $p = 0.0112$) and between the categories

($F = 80.3921$, $P < 0.0001$). The average number of intact seeds differed between the cytotypes, with diploids showing higher numbers than tetraploids ($p = 0.0027$). When considering the three categories, the average number of intact seeds in diploids and tetraploids was greater than those of aborted seeds in diploids and tetraploids (in both $p = 0.0001$ and $p = 0.0001$ respectively) and higher than the average number of predated seeds in diploids and tetraploids ($p = 0.0001$) (Fig. 3). The Pre-Emergent Reproductive Success of diploids was 2.5-times higher than that of tetraploids (Table 3).

Discussion

The floral traits of *L. ferrea*, such as diurnal anthesis, sweet odour and yellow zygomorphic flowers with a nectar guide, suggest that the species is melittophilous (sensu Faegri and Pijl 1979). In fact, the effective pollinators of this species
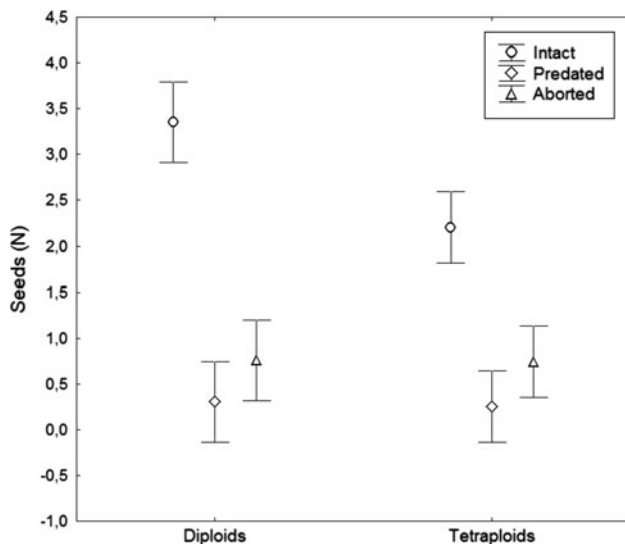
Fig. 3 Mean number of intact, predated and aborted seeds per fruit in diploid and tetraploid individuals of *Libidibia ferrea* occurring in Pernambuco State, northeastern Brazil

were medium- to large-sized bees. In this respect, *L. ferrea* does not differ from the most common pattern observed for Leguminosae or *Caesalpinia* s.l., the genus in which *L. ferrea* was placed until the recent resurrection of *Libidibia*. Both are predominantly melittophilous (Arroyo 1981; Borges et al. 2009; Leite and Machado 2009), although both the family and the genus have several examples of adaptive radiation in their floral syndromes, with species that are pollinated by birds, bats, butterflies and hawkmoths (Arroyo 1981; Vogel 1990; Lewis et al. 2000; Borges et al. 2009). Another feature presented by *L. ferrea*, which is widespread in the genus *Caesalpinia*, is the variation in pistil size in different flowers of the same plant, with some species being considered andromonoecious (Gibbs et al. 1999; Borges et al. 2009). In *C. calycina*, for example, most of the flowers analysed by Lewis and Gibbs (1999) consisted of hermaphroditic flowers at the base of the inflorescence, and flowers with aborted ovaries or reduced pistils at the apex of the axis. According to these authors, the function of andromonoecy in the species would be an increase in male function, and, in fact, Gibbs et al. (1999) found that the labile sex change of flowers in *C. calycina* was dependent upon successful fertilization of, and fruit set in, the lower flowers. If fruits were not set lower down the inflorescence then the upper flowers retained hermaphroditic status (Gibbs et al. 1999). Nevertheless, although *L. ferrea* presented some variation in pistil size, the species is clearly hermaphroditic, since the frequency and position of flowers with reduced or stunted pistils seemed to be low and random, respectively. It is possible that *L. ferrea*, as well as other species of *Caesalpinia* s.l., presents the

innate ability required for the evolution of andromonoecy. However this sexual system is not established yet in *L. ferrea* and in other species of the genus.

Although the flowers of diploids and tetraploids varied greatly in size and in other features of morphology, both cytotypes had the same effective pollinators. Therefore, in the case of mixed populations or adjacent diploid and tetraploid populations, flower visitors, theoretically, may promote substantial pollen flow between cytotypes. This interploidy pollen transfer could potentially produce triploid zygotes (unviable or sterile), which would be particularly disadvantageous to the minority cytotype (Levin 1975). There may be more subtle differences in the relative frequencies of each pollinator, as has been observed between diploid and tetraploid cytotypes of *Heuchera grossulariifolia* in the Saxifragaceae (Segraves and Thompson 1999), although only a detailed analysis of the frequency of visits will clarify this. These differences could contribute to a reproductive niche separation between cytotypes and counterbalance the negative effects on the minority cytotype. Another condition that could promote such niche separation would be the differential deposition of pollen from diploids and tetraploids on a bee's body. In fact, there was a significant difference between the landing platform lengths in both cytotypes, which was approximately 15 % larger in the flowers of tetraploids compared to those of diploids (Table 1). This difference may be enough to decrease the rate of pollination between cytotypes and promote niche separation between *L. ferrea* diploids and tetraploids.

With respect to the breeding system, our study has shown that there was a difference between cytotypes, with diploids being self-incompatible and tetraploids being self-compatible. This difference is an example of one of the consequences of polyploidy—the breakdown of self-incompatibility (Levin 2002)—and may be important for the establishment and dissemination of polyploids (Jackson 1976). Selfing ability may have been one of the factors that allowed the success of *L. ferrea* tetraploids, which even after cross-pollination had low fruit-set, especially when compared with the diploids.

One issue that is worth mentioning is the species' low reproductive success, particularly in the tetraploid individuals. High flower and fruit abortion rates in natural conditions are a common phenomenon in plants and have attracted the attention of researchers for some time (Bawa and Webb 1984). Although we have not quantified the factors that may be responsible for this characteristic in *L. ferrea*, some inferences can be made and are outlined in the discussion below.

The mass flowering that begins in the dry season renders the trees of *L. ferrea* susceptible to frequent geitonogamous pollinations since bees are likely to forage for a while at

flowers within a single canopy. For self-incompatible diploids, this is particularly disadvantageous and could be reflected in the low Reproductive Efficacy observed. On the other hand, the low fruit production under natural conditions that was also observed in self-compatible tetraploids requires a different explanation, particularly since this cytotype formed few fruits after hand cross-pollinations. Thus, the lack of adequate pollination cannot be considered as a significant factor for the low reproductive success of this species, at least not for the two cytotypes. Bawa and Webb (1984) also dismissed this hypothesis to explain ovule abortion at least in the four species of Leguminosae they studied. One factor that would result in lower reproductive success would be a lower viability of the tetraploid gametes due to polyploidy. However, as observed in this study, pollen viability was high and did not differ between the two cytotypes, and Beltrão and Guerra (1990) observed regular meiosis in the tetraploids, which would imply that gametes formed normally.

Florivory by the larvae of Lepidoptera, which principally damage the plant's female organs, is found in both cytotypes and could be a factor that influences the high rate of flower abortion observed in this species. This possibility was also considered by Bawa and Webb (1984) as a possible cause of excessive flower production. It would be interesting to investigate the proportion of affected flowers in the two cytotypes and evaluate the rate of fruit formation in flowers with and without florivores.

The absence of fruit after interploidy crosses indicates that there is no gene flow between the two cytotypes, or, if there is, the levels are very low. Therefore, we conclude that *L. ferrea* diploids and tetraploids are reproductively isolated. This statement, together with the other findings discussed above, has important consequences for the ecology and taxonomy of the species and for conservation and reforestation plans.

First, the exclusion of the minority cytotype is expected to adversely affect tetraploids in mixed *L. ferrea* populations. Although this cytotype has shown a breakdown in self-incompatibility, the low fruit-set after a priori compatible crosses is a major disadvantage in comparison to the diploids. As there was no complete separation of reproductive niche in cases where both cytotypes occur in sympatry, the same pollinators are shared. Thus, arguments that explain the establishment of many tetraploid populations need to be based not on the replacement of diploids by tetraploids, the analysis of which dominates the study of polyploid establishment (see the "Introduction"), but on mechanisms for dispersal and colonisation of new habitats, as suggested by Thompson and Lumaret (1992) and Fawcett and Van de Peer (2010). The latter authors emphasised the ability of many tetraploids to colonise harsh environments, where their diploid progenitors were

unable to establish. In this context, the distribution of *L. ferrea* is interesting: while the diploid populations occur almost exclusively in dry forests near the coast or in areas of Caatinga at higher elevations in the interior of the state, both of which have mild weather, the tetraploids occur almost exclusively in areas of Caatinga with more severe weather conditions (Beltrão and Guerra 1990, unpublished data). In these places there are distinct seasons, including a very severe dry season during which all vegetation loses its leaves (Queiroz 2009). Because the fruits are zoochoric (primarily barochoric/secondarily zoochoric) (L. Borges, personal observation), it is possible that the tetraploids have been able to quickly establish viable populations in these locations, despite their very low reproductive success.

With cytotypes that are reproductively isolated and that display great morphological variability, as shown by the existence of four described varieties within the species, as well as the floral morphometric results presented here, a revision of the limits of *L. ferrea* and the possible proposal of new taxa that are more consistent with the biological reality of the species is critical. Soltis et al. (2007) noted that there are only a few cases in which autopolyploids have been treated as separate species from their diploid progenitors. However, these authors suggested that this tradition should be changed because it does not reflect the variety of biological entities in nature, and they also argued that the goal is not to name all autopolyploids, but to carefully examine such cases before considering them as mere cytotypes, because they often meet the requirements of various species concepts. An important contribution of the present study in initiating this task is the evidence that *L. ferrea* diploids and tetraploids are reproductively isolated and have different floral morphometries, which allowed us to deduce diagnostic morphological characteristics for the two cytotypes.

In conclusion, we would like to emphasise the importance that this knowledge may have for conservation planning. Although *L. ferrea* is not endangered, its use in reforestation projects, such as the one currently in progress on the banks of the São Francisco River, must be carried out with caution and care. If diploid and tetraploid matrices are used indiscriminately for the production of seedlings that are planted in the same areas, it is possible to create an inadequate situation for the recovery of those areas. The artificial populations could face imbalances caused by decreased reproductive success, which is already low, through interbreeding between the different cytotypes. In addition, if physiological and ecological differences favour the establishment of a single cytotype, many seedlings will perish because they are not able to establish under those conditions, which will reduce the effective population size.

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